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UNIVERSITY OF ALBERTA

CHARACTERIZATION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS AND THEIR MODULATION BY PARATHYROID HORMONE IN THREE TYPES OF CELLS

BY

RUI WANG

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF **DOCTOR OF PHILOSOPHY**

DEPARTMENT OF PHYSIOLOGY
EDMONTON, ALBERTA

SPRING, 1991

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DEDICATION

To my dear wife Lingyun Wu, my lovely daughter Jennifer Wang,
and my parents, Zhenyuan Li and Chunmin Wang.

ABSTRACT

Beside its traditional hypercalcaemic action, parathyroid hormone (PTH) also produces vasodilatory and cardiotonic effects which are extracellular calcium dependent. In addition, PTH has been reported to affect the transmembrane calcium movement in synaptosomes obtained from rat brain. The occurrence of PTH-like immunoreactivity has also been demonstrated in the central nervous system (CNS). Therefore, this endogenous circulating peptide may play an important role in the modulation of voltage- and time-dependent calcium channels (VDCC) in both the cardiovascular and nervous systems.

In the present study the whole-cell version of the patch clamp technique was used to identify the two types of VDCC in three cell preparations. The separation of the transient (T) channel current from the long-lasting (L) one was achieved in N1E-115 cells by the use of different culture media. In this way, predominantly T or L channel currents could be expressed selectively. In rat tail artery smooth muscle cells, T and L channel currents were identified. Their existence had not been previously reported in cells from this particular vessel. The two types of VDCC in neonatal rat ventricular myocytes were also characterized in detail in order to extend our knowledge of this cell preparation.

The active synthetic bovine PTH peptide, bPTH-(1-34), used in the present study was demonstrated to have no effect on T channel currents in these three cell preparations. However, bPTH-(1-34) inhibited L channel currents in N1E-115 cells

and rat tail artery smooth muscle cells without any change in the normalized conductance curve, while L channel currents in neonatal rat ventricular myocytes were enhanced with a negative shift in the normalized conductance curve. These effects of bPTH-(1-34) were concentration-dependent. An inactivated (oxidized) preparation of bPTH-(1-34) had no effect on L channel currents in N1E-115 cells. bPTH-(3-34), by itself, had no effect on L channel currents in vascular smooth muscle or ventricular muscle cells. However, pretreatment of cells with bPTH-(3-34) abolished the effect of subsequently applied bPTH-(1-34) on L channel currents. The kinetics of L channel currents were not changed by bPTH-(1-34) in vascular smooth muscle cells or N1E-115 cells. However, bPTH-(1-34) decreased the rate of activation and bPTH-(3-34) increased the rate of inactivation of L channel currents in ventricular myocytes. The exposure of these myocytes to bPTH-(3-34) prior to the application of bPTH-(1-34) also eliminated the changes in the activation rate of L channel currents induced by bPTH-(1-34). Wash-out studies revealed that the effect of bPTH-(1-34) on L channel currents was reversible within 5 min in vascular smooth muscle cells but not in ventricular myocytes. The effects of bPTH-(1-34) on the contraction of single vascular smooth muscle cells and ventricular myocytes were also studied. bPTH-(1-34) inhibited KCl-induced contraction of single smooth muscle cells but resulted in contraction in ventricular myocytes. These results of the effects of bPTH-(1-34) on cell contraction further suggest the functional relevance of bPTH-(1-34) effect on L channel currents observed in patch clamp studies.

It was also found, in the present study, that dibutyryl cAMP (db-cAMP), when applied to the bath solution, mimicked the effects of bPTH-(1-34) on L channel currents in both smooth muscle cells and ventricular myocytes. Sequential application of db-cAMP and bPTH-(1-34) did not lead to a synergistic increase in L channel currents in ventricular myocytes, or a synergistic decrease in smooth muscle cells. The lack of the synergistic effects of db-cAMP and bPTH-(1-34) indicated that these two agents acted on the same final sites. Furthermore, the intracellular infusion of 100 μM Rp-cAMPs, a cAMP antagonist, prior to the application of bPTH-(1-34) totally abolished the effects of bPTH-(1-34) on L channel currents in both vascular smooth muscle cells and ventricular myocytes.

PTH is the first identified endogenous peptidergic VDCC modulator which has opposite effects on L channel currents in vascular smooth muscle cells and ventricular myocytes.

Specific Aims

1. Characterization of voltage-dependent calcium channel currents in neuroblastoma cells, vascular smooth muscle cells and ventricular myocytes (Chapters 3,4,5).

2. Investigation of the effects of PTH on voltage-dependent calcium channel currents in neuroblastoma cells, vascular smooth muscle cells and ventricular myocytes (Chapters 6,7,8,9).

3. Delineation of the mechanisms of PTH effects on voltage-dependent calcium channel currents in vascular smooth muscle cells and ventricular myocytes, especially the role of cAMP in the modulation of PTH effects on voltage-dependent calcium channel currents (Chapter 10).

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List of Abbreviations

Ach	acetylcholine
AVP	arginine vasopressin
cAMP/cyclic AMP	adenosine 3':5'-cyclic monophosphate
cGMP/cyclic GMP	guanosine 3':5'-cyclic monophosphate
CGRP	calcitonin gene-related peptide
CICR	Calcium-induced calcium release
CNS	central nervous system
D-600	methoxyverapamil
db-cAMP	dibutyryl cyclic AMP
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
d.r.g.	dorsal root ganglia
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
FCS	fetal calf serum
G proteins	guanine-nucleotide-binding proteins
HBSS	Hanks' Balanced Salt Solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
L channel	long-lasting calcium channel
NA	noradrenaline
NE	norepinephrine

PKC	protein kinase C
PTH	parathyroid hormone
bPTH	bovine parathyroid hormone
hPTH	human parathyroid hormone
PTHLPs	PTH-like proteins
Protein kinase A	cyclic AMP-dependent protein kinase
Rp-cAMPs	adenosine 3':5'-cyclic monophosphothioate, Rp - isomer
SMC	smooth muscle cells
SR	Sarcoplasmic reticulum
T channel	transient calcium channel
TEA-Cl	tetraethylammonium chloride
Tris	tris(hydroxymethyl)aminomethane
TTX	tetrodotoxin
VDCC	voltage- and time-dependent calcium channels

CHAPTER 1

Introduction and Literature Review

Ion channels are macromolecules which span the plasmalemma to provide an aqueous route for the passage of ions. The charged character of ions would otherwise prohibit their movement through the membrane's lipid environment. Ion flux through such channels is a passive process, which requires only that channels are open for ions to descend electrochemical gradients into or out of the cell. At least three types of changes can open ion channels, i.e. changes in membrane potential (voltage-dependent channels); occupation of channel-associated receptors by drugs, neurotransmitters, or hormones (receptor-operated channels); or changes in the surface area of the cell membrane (stretch-activated channels).

Among the most widely distributed and important ion channels, voltage- and time-dependent Ca^{++} channels, **which are referred to as calcium channels in this thesis**, are activated by membrane depolarization. Multiple types of calcium channels have been identified in different excitable and non-excitable cells. The criteria for classifying calcium channels are the single-channel properties, voltage- and time-dependence, ionic selectivity and pharmacological sensitivity.

The pharmacological modulation of calcium channels by synthetic compounds is very important in both their characterization and purification, and therapeutic use. In addition, the modulation of these channels by endogenous hormones and neurotransmitters plays a pivotal role in many important physiological processes. Recently, it has been demonstrated that endogenous agents can increase or decrease calcium channel currents in various excitable cells. Many facets of the modulation of calcium channels by these endogenous agents, such as the channel specificity, tissue selectivity and the role of second messengers, have not been completely elucidated.

Multiple target tissues of parathyroid hormone (PTH) have been identified. In addition to bone, kidney and gut, vascular smooth muscle and cardiac muscle can also respond to PTH (see review by Mok *et al.*, 1989a). Although it has been suggested that PTH-induced cardiovascular effects are mediated, at least partly, by the influx of calcium ions (Pang *et al.*, 1988a; Bogin *et al.*, 1981), direct electrophysiological evidence at the single cell level is not available. Both the cardiovascular effects of PTH and activities of calcium channels in myocardial cells have been shown to be modulated by cAMP. It is, therefore, important to determine whether cAMP mediates the cardiovascular effects of PTH and the modulation of calcium channels.

In this chapter, the classification of calcium channels in neurons, vascular smooth muscle cells and cardiac myocytes, the cardiovascular and neuronal effects of PTH and its underlying mechanisms, and the role of cAMP in the mediation of calcium channels and PTH effects are reviewed. Extending from this literature review, the objectives of this thesis are stated.

1. Voltage-dependent calcium channels

1.1. Neuronal cells

1.1.1. Classification of calcium channels

Multiple subtypes of calcium channels have been identified in neurons (Bossu *et al.*, 1985; Nowycky *et al.*, 1985; Penner and Dreyer, 1986; Fox *et al.*, 1987). In neurones of the dorsal root ganglion (d.r.g.) (Nowycky *et al.*, 1985), three types of calcium channels have been reported. Long-lasting (L) channels are activated by strong depolarizations, inactivate slowly and are highly sensitive to Cd^{++}. Ba^{++} is more permeable than Ca^{++} through L type calcium channels. In contrast, the transient (T) channels are activated by weak depolarizations, inactivate quickly and are less affected by Cd^{++}. The T channel has a similar permeability to Ba^{++} and Ca^{++}. A third type of calcium channel, the neuronal (N) channel, differs from L channels since this macroscopic current is transient inactivated as well as resistant to modulation by dihydropyridines. The N channel differs from T channels in that it is activated by strong depolarizations and blocked by a low concentration of Cd^{++}. N channels are present only in certain neurones, and are localized at motor nerve terminals, cell bodies and growth cones of sympathetic neurons. L channels, however, are predominantly localized on cell bodies (Tsien *et al.*, 1988). In sympathetic neurons, such as rat superior cervical ganglion neurons and bullfrog sympathetic neurons, only N and L channels are identified. T channel currents are suggested to be absent (Miller *et al.*, 1988).

Different types of calcium channels can also be distinguished by their pharmacological sensitivities. L channel currents are sensitive to dihydropyridines, such as nifedipine and Bay K-8644. However, relative high concentrations of dihydropyridines are needed to affect neuronal L channels as compared to other tissues (Miller *et al.*, 1988). ω-C_gT_x (conotoxin) can block both N and L channels in neurons. However, N channels are peculiar in their insensitivity to dihydropyridines and sensitivity to Cd^{++}, respectively (Dascal, 1990). Traditionally, T channels were believed to be resistant to dihydropyridines. However, felodipine has been reported to block both T and L channels (Van Skiver *et al.*, 1989). Other agents used to block neuronal T channel currents include 1-octanol (Llinas, 1988), amiloride (Tang *et al.*, 1988), chlorpromazine (Ogata and Narahashi, 1990) and nicergoline (Takahashi and Akaike, 1990).

Using the patch clamp technique (Hamill *et al.*, 1981), Narahashi *et al.* (1987) demonstrated the existence of two types of calcium channels in neuroblastoma N1E-115 cells. One advantage in using N1E-115 cells is the easy electrophysiological separation of the T channel currents from the L channel currents. Hence, N1E-115 cells represent a useful model for studying different types of calcium channels, and examining the effects of drugs and hormones in a homogeneous system where biochemical, physical and electrophysiological measurements can be correlated (Freedman *et al.*, 1984a; Quandt and Narahashi, 1984). N1E-115 cells contain tyrosine hydroxylase which catalyses the formation of L-Dopa, the first step in norepinephrine synthesis (Amano *et al.*, 1972). In this regard, N1E-115 cells (Tsunoo *et al.*, 1986) can be utilized as a model to study the effects of PTH or other hormones and drugs on neurotransmitter release from nerve terminals.

1.1.2. Physiological functions of calcium channels in neurons

One of the most important roles of Ca^{++} channels in neural cells is to provide a route for the entry of Ca^{++} into nerve terminals to effect neurotransmitter release. Neurotransmitter release is specifically dependent on Ca^{++} entry rather than on depolarization alone (Zucker and Lando, 1986). Ultrastructural studies of the neuromuscular junction have indicated that neurotransmitter vesicles are arranged along specialized regions (active zones) of the presynaptic membrane, which contain electron-dense particles (Atwood and Lnenicka, 1986). It has been proposed that active-zone particles represent voltage-dependent Ca^{++} channels (Pumplin *et al.*, 1981).

N and L channels both require relatively strong depolarizations for opening and may be considered as contributors to "high-voltage-activated" neuronal functions such as dendritic spiking or neurotransmitter release.

Dihydropyridine-sensitive L channels are found in all neurons. Their voltage dependency and kinetics seem well-suited for transforming the time integral of membrane depolarization into an intracellular Ca^{++} signal for triggering the cellular response. One example of such voltage-response transduction is the release of substance P from d.r.g. neurons (Rane *et al.*, 1987).

The distribution of N channels has received less attention than the distribution of the other types of calcium channels since the discovery of this channel is relatively recent. Nevertheless, available data is consistent with the hypothesis that the expression of N-type channels may be limited to neuronal or neurone-like membranes. Hence, N channels possess the properties of Ca^{++} entry pathways underlying transmitter release from sympathetic neurones (Hirning *et al.*, 1986), motor nerve terminals (Quastel *et al.*, 1986) and synaptosomes (Reynolds *et al.*, 1986).

T channels are found along with L channels in a wide variety of neurons. The voltage-dependence of T channels makes them a logical candidate for generation of spontaneous depolarizations and for rebound excitation following strong hyperpolarization. In addition, T channel activation may be important in determining basal levels of hormone secretion (Cohen and McCarthy, 1987) and for initiating near-threshold membrane phenomena, e.g. speeding up depolarization to threshold after neuronal hyperpolarization (Yaari *et al.*, 1986). The resting potential of N1E-115 cells ranges from -35 to -55 mV (Moolenaar and Spector, 1978) which is the voltage range of activation for T channels, so that T channels probably activate near the resting potential of the cell. In some neurons, the T channels contribute more than L channels to the total Ca^{++} currents moving through the cell membrane (Carbone and Lux, 1986).

1.1.3. Effects of neuropeptides and hormones on calcium channels in neurons

In neuronal preparations, enhancement of the inward Ca^{++} current has been observed in response to peptides such as substance P (Murase *et al.*, 1986), calcitonin and calcitonin gene-related peptide (Nohmi *et al.*, 1986). A variety of excitatory amino acids, including putative neurotransmitters, have also been shown to stimulate Ca^{++} influx into brain slices and cultured neurons (Berdichevsky *et al.*, 1983; Wroblewski *et al.*, 1985), although it remains unclear whether the Ca^{++} channel(s) that mediate these effects are voltage-dependent. Other neuromodulators inhibiting calcium channels include dopamine (Paupardin-Tritsch *et al.*, 1985; Marchetti *et al.*, 1986), serotonin (Dunlap and Fischbach, 1981), gamma-aminobutyric acid (GABA) (Dunlap and Fischbach, 1981), the kappa-opioid peptides dynorphin and neoendophin (Werz and MacDonald, 1985a), somatostatin (Dunlap and Fischbach, 1981; Tsunoo *et al.*, 1986), adenosine (Macdonald *et al.*, 1986) and prostaglandin E_1 (Mo *et al.*, 1985). The inhibition was reported to be specific for L channels in NG108-15 cells by somatostatin (Tsunoo *et al.*, 1986), for N channels in hippocampal neurons by adenosine (Madison *et al.*, 1987), and for T channels in *Aplysia* neurons by FMRF amide (Kramer *et al.*, 1988). However, in chick d.r.g. neurons the inhibition of T and L channels by dopamine has also been observed (Marchetti *et al.*, 1986).

The most completely understood transmitter effect is an inhibition of calcium current in sensory neurons produced by α-adrenergic agonists (Dunlap and Fischbach, 1981), an effect that probably underlies a form of adrenergic presynaptic inhibition. Norepinepherine inhibited both T and L channel currents in d.r.g. neurons (Bean, 1989a).

Monosynaptic facilitation of transmitter release has been found in many synapses (Hoshi *et al.*,1984). One possible mechanism for this synaptic facilitation is that a specific calcium channel, i.e. facilitation calcium channel, is activated by pre-depolarizations. Due to the inaccessibility of most presynaptic terminals, studies of facilitation calcium channels have been carried out in some cases in neurosecretory cells such as chromaffin cells (Artalejo *et al.*, 1990). In bovine chromaffin cells, Artalejo *et al.* (1990) have suggested that the release of dopamine activates the D_1 receptor which in turn activates adenylate cyclase. The resulting increase in cAMP activates protein kinase A which in turn activates facilitation calcium channels. This cascade of events may form a positive feedback mechanism which augments catecholamine release. This data suggests that in some cells of neuronal origin facilitation calcium channels exist in addition to regular calcium channels. These neuronal facilitation calcium channels may be modulated by physiological stimuli in order to regulate the level of cell activation.

1.2 Vascular smooth muscle cells

1.2.1. Classification of calcium channels

In smooth muscle cells, calcium influx through the voltage-dependent calcium channels plays an essential role in the regulation of excitation-contraction coupling (Yoshino and Yabu, 1985; Droogmans and Callewaert, 1986). As early as 1969, Anderson began to analyze inward calcium current in uterine smooth muscle strips. Since then, our knowledge of calcium channels has expanded due to research using different types of multicellular smooth muscle tissues and voltage clamp techniques. However, spatial non-homogeneity of voltage control during a clamp step as well as the presence of a complex and large outward potassium current in the above tissues impeded studies of the voltage-dependent calcium channels in smooth muscle cells. The combination of the patch clamp recording technique (Hamill *et al.*, 1981) with single smooth muscle cell isolation techniques has removed some of these obstacles. As a result, it has become clear that there are two types of calcium channels coexisted in same smooth muscle cells, each with distinct features (Reuter, 1985).

Both T and L types of calcium channels have been identified in different vascular smooth muscle cells, such as those from mesenteric artery (Bean *et al.*, 1986; Ohya and Sperelakis, 1989), ear artery (Benham *et al.*, 1987b) and portal vein (Ohya *et al.*, 1988). However, L channels are still the main population of calcium channels in vascular smooth muscle cells, and are more important than T channels under physiological conditions (Ohya and Sperelakis, 1989). Using the patch clamp technique, Toro *et al.* (1986) recorded only K^+ channel currents but not any inward currents in rat tail artery smooth muscle cells. These authors suggested the absence of Ca^{++} channels in this cell preparation. This report differs from the hypotheses of other investigators since the results from tension assays and $^{45}Ca^{++}$ uptake in this tissue suggested the existence of calcium channels (Pang *et al.*, 1985b, 1988a).

Bolton *et al.* (1985) have demonstrated the inward Ca^{++} current in whole-cell recording in guinea-pig and rabbit mesenteric artery smooth muscle cells, but they failed to demonstrate the existence of single calcium channel currents using inside-out patch configuration. This is in agreement with the observation made by Ohya *et al.* (1987) in which T-type Ca^{++} channels retained their functional activity in isolated membrane patches, whereas, L-type calcium channels deteriorated rapidly.

To recapitulate, the presence of two kinds of Ca^{++} channels has been established in vascular smooth muscle cells. T channels have a smaller population than do L channels. Different from neurons, a faster inactivation of L channel currents is often observed in vascular smooth muscle cells. The current densities of both T and L channels are smaller in vascular smooth muscle cells than in neurons or cardiac muscle cells. Therefore, the recording of these smooth muscle calcium channel currents is more difficult. The stability of Ca^{++} currents differs in various smooth muscle cells. Comparisons between results of cell-attached and cell-free patch clamp recordings reveal that the "run down" of calcium currents in smooth muscle cells is also due to a loss of some components of the biochemical machinery of the cell interior, which keeps calcium channels active.

1.2.2. Physiological functions of calcium channels

The diversity of Ca^{++} channels in smooth muscle cells may correspond to a variety of cell functions. Sustained (L-type) Ca^{++} channels probably supply Ca^{++} for contractile protein activation and intracellular storage sites, especially in resistance arteries (van Breemen *et al.*, 1987; Goldman *et al.*, 1989). Different types and ratios of Ca^{++} channels may, thus, be present in various smooth muscle cells and account for their excitation-contraction coupling characteristics (Sturek and Hermsmeyer, 1986). The function of T channels in vascular smooth muscle cells is, to date, not clear. Smooth muscle cells of larger blood vessels do not generate an action potential when they are depolarized. However, when an excitatory junction potential exceeds the threshold in response to nerve stimulation, action potentials can be elicited in smooth muscle cells of small arteries, such as the rabbit ear artery (Droogmans *et al.*, 1977). Both T and L channels may contribute to the generation of action potentials in vascular smooth muscle cells.

1.2.3. The modulation of calcium channels by hormones and neurotransmitters.

The best known examples of endogenous modulators of calcium channels are noradrenaline (NA) and endothelin.

In rabbit ear artery smooth muscle cells, NA increased L channel currents in a concentration- and time-dependent manner. This effect of NA is reversible and not mediated by α- or β- adrenoceptors. The inclusion of GTP in the pipette solution enhanced the effect of NA on L channel currents. G proteins may be the coupling factor between the binding of NA and the increase in L channel currents (Benham and Tsien, 1988). The increase of calcium channel currents in single smooth muscle cells from rabbit mesenteric artery by NA was further confirmed at the single channel level (Nelson *et al.*, 1988). However, Droogmans *et al.* (1987) reported that NA inhibited L channel currents in the same cell preparation and under the same experimental conditions as in the report by Benham and Tsien (1988). This inhibition of L channel currents by NA was explained as an activation of α_1-receptors. The reason for the difference between these results has still not been resolved.

Endothelin, a twenty-one amino acid peptide, is, thus far, the most potent naturally occurring vasoconstrictor ($EC_{50} = 4 \times 10^{-10}$ M) to be identified (Yanagisawa *et al.*, 1988). Results from patch clamp studies (Goto *et al.*, 1989; Silverberg *et al.*, 1989) indicated that endothelin activated voltage-dependent calcium channels in vascular smooth muscle cells. In smooth muscle cells from guinea-pig portal vein, endothelin enhanced both the nifedipine-sensitive and resistant calcium channel currents (Inoue *et al.*, 1990). Interestingly, endothelin-induced augmentation of calcium channel currents required a period of more than 5 min after the addition of the peptide to the bath for full development. Furthermore, wash-out did not fully reverse the endothelin effect. In addition, intracellular application of endothelin was less potent in increasing calcium channel currents than was extracellular application. These observations lead Inoue *et al.* (1990) to suggest that the effect of endothelin on calcium channels was mediated by second messengers. The stimulation of protein kinase C was proposed as one possible mechanism (Resnik *et al.*, 1988). Forder *et al.* (1985) reported that the activation of protein kinase C led to the contraction of vascular smooth muscle cells by increasing calcium permeability. In contrast, other studies indicated that the activation of protein kinase C inhibited calcium channels. For example, inward calcium channel currents in the A7r5 cell line from an embryonic rat aortic muscle was inhibited by 10 nM vasopressin (Galizzi *et al.*, 1987). The mechanism underlying the vasopressin effect was postulated to be the following: vasopressin activates phospholipase C which catalyses the formation of diacylglycerol; protein kinase C is subsequently activated.

1.3. Calcium channels in myocardial cells

1.3.1. Classification of calcium channels

The first description of an L-type calcium channel in cardiac tissue was published in 1967 by Reuter. Since the sucrose gap method was the best available technique at that time, the calcium conductance in cardiac tissue was simply described as a single population of channels. In 1985, Nilius and co-workers reported two types of voltage-dependent calcium channels in cardiac cells. The identification of T and L channels was confirmed using the whole-cell version of the patch clamp technique (Bean, 1985; Mitra and Morad, 1986). The dihydropyridine-sensitive L channels are the predominant component in adult cardiac myocytes. The cardiac T channel is not sensitive to dihydropyridine, but has been reported to be sensitive to nickel and tetramethrin (Hagiwara *et al.*, 1988). The relative magnitude of the T current is small in adult ventricular cells, larger in atrial cells, and largest in natural pacemaker cells (Tsien *et al.*, 1987a, b). However, a larger population and density of T channels than L channels has been reported in embryonic chick ventricular myocytes (Kawano and DeHaan, 1990). T and L channel currents have different densities in different tissues. In addition, the steady-state inactivation of L channels is different in vascular smooth muscle and cardiac muscle cells. When the holding potential was shifted from -80 to -40 mV, the L channel current was inactivated by approximately 50% in smooth muscle cells but less than 10% in atrial cells (Bean, 1989a, b). The dihydropyridine sensitivity of L channel currents is also different in cardiac cells and neurons. The peak of the I-V relationship of L channels was shifted toward the negative direction by Bay K-8644 in cardiac cells, but not in neurons (Wang *et al.*, 1990a).

The third type of calcium channel was discovered by Rosenberg and Tsien (1987) in cardiac sarcolemmal membranes. This channel opened at the normal resting potential. Later, this background calcium conductance or B channel was confirmed using the single channel recording technique in adult rat ventricular myocytes (Coulombe *et al.*, 1989). B channel currents were only detected at negative potentials. This channel was not sensitive to inorganic blockers, such as cobalt, cadmium or nickel. Bay K-8644 increased B channel currents.

1.3.2. Physiological function of calcium channels

Calcium currents appear to be highly significant for the generation of the latter part of diastolic depolarization and the upstroke of the action potential in the mammalian sinoatrial nodal preparation (Noma *et al.*, 1983). The initial phase of the pacemaker potential is generated mainly by a decaying conductance of the delayed K^+ current but the latter phase is produced by an activation of T channel currents. It is also hypothesized that an increase in L channel currents but not T channel currents triggers the upstroke of action which is responsible for the isoproterenol induced positive chronotropic action (Hagiwara *et al.*, 1988). Since T channels have not been found in the majority of adult ventricular myocytes, it is suggested that T channels are not essential for the normal working myocardium. B channel currents might be important for the resting calcium influx.

1.3.3. The modulation of calcium channels by hormones and neurotransmitters

β-adrenergic agonists enhance the calcium current in cardiac muscle cells (Reuter, 1967) and this effect is directly involved in the increased rate and strength of the heart beat that is produced by sympathetic stimulation.

Isoproterenol, a β-adrenergic agonist, enhances the high-threshold and low-threshold calcium channel currents in guinea pig ventricular myocytes (Mitra and Morad, 1986). It is known that the formation of the transmitter- β-receptor complex activates adenylate cyclase, which in turn increases cAMP content. Then, cAMP phosphorylates a channel protein through cAMP-dependent protein kinase, and opens channels (Tsien *et al.*, 1986).

Using whole-cell recording, Binah *et al.* (1987) found that thyroid hormone increased calcium channel currents in guinea pig ventricular myocytes. It is not known whether this effect is induced by direct action of thyroid hormone on the channel protein, or indirectly via an intracellular second messenger.

The enhancement of calcium channel currents by histamine was demonstrated in guinea pig ventricular myocytes (Hescheler *et al.*, 1987a). A mechanism of cAMP-dependent phosphorylation of calcium channels was suggested to be responsible since the effect of histamine could be abolished by Rp-cAMPs, a blocker of the cAMP-dependent protein kinase.

Ono *et al.* (1989) reported that calcitonin gene-related peptide enhanced calcium currents in frog and rabbit atrial myocytes. This effect was reversible and mediated by cAMP. A novel endogenous peptide with a low molecular weight, isolated and purified from rat brain, increased L channel currents in guinea pig ventricular myocytes (Callewaert *et al.*, 1989).

2. The cardiovascular effect of PTH and the underlying mechanisms

2.1. The hypotensive effect of PTH

Although the hypercalcemic effect of PTH has been studied in detail and firmly established, another important action of PTH, i.e. PTH-induced cardiovascular effect, has not been widely recognized until recently. The hypotensive and vasodilatory action of PTH was initially reported by Collip and Clark in 1925, and then by Handler and Cohn in 1952, and Charbon in 1966. After a series of carefully designed and executed experiments, Pang and his co-workers (1988a), as well as other investigators (see review by Mok *et al.*, 1989a), demonstrated that PTH was indeed a vasodilator. This statement is supported by several lines of evidence. 1) PTH induced hypotension could be elicited in many different species of vertebrates including rat, dog, fish, bullfrog, water snake, chicken (Pang *et al.*, 1980a, b) and cat (Lepak *et al.*, 1987). 2) The hypotensive action is a property of the PTH molecule itself rather than some contaminant (Pang *et al.*, 1985a). 3) The action of PTH produces a direct effect on blood vessels, which is not mediated by other vasoactive substances such as histamine or acetylcholine (Pang *et al.*, 1980a; Yang *et al.*, 1981), nor by adrenergic mechanisms (Pang *et al.*, 1981). The vasoactive effect of PTH is, in addition, not dependent on an intact endothelial cell layer (Pang *et al.*, 1985b). 4) PTH is more effective in relaxing resistance-type vessels than conduit vessels. Rat tail artery (Pang *et al.*, 1985b) and dog coronary artery (Crass and Pang, 1980) were very sensitive to PTH, while the rat and rabbit aortae were rather insensitive (Pang *et al.*, 1984). 5) The vasodilatory action of PTH is related to the structure of this peptide molecule (Pang *et al.*, 1985a). The amino acids of PTH in positions 1 (serine/alanine for human/bovine) and 2 (valine) are essential for the vascular action of PTH. Hydrogen peroxide oxidation essentially destroyed the vascular action of PTH (Kenny and Pang, 1982). 6) The effect of PTH on blood pressure is not secondary to decreased cardiac output (Berthelot and Gairard, 1975), since PTH, by itself, increased cardiac output. Wang *et al.* (1984) reported that bovine PTH [bPTH-(1-34)] decreased arterial blood pressure and total peripheral resistance, and increased cardiac output in a concentration-dependent fashion.

Further studies by Pang *et al.* (1988a) demonstrated that PTH could act as a calcium entry blocker in vascular tissues. In rat tail artery tension studies, bPTH-(1-34) inhibited the vasoconstriction produced by 60 mM KCl. KCl has been shown to produce depolarization of vascular smooth muscle cells and thereby open the voltage-dependent calcium channels in the membrane. In the presence of bPTH-(1-34), the tissue contraction stimulated by Bay K-8644 was significantly reduced. Bay K-8644 is a specific agonist of the long-lasting type of calcium channels. Furthermore, bPTH-(1-34) significantly decreased the KCl-stimulated $^{45}Ca^{++}$ uptake in the rat tail artery tissues. The effects of PTH in these two assays were extracellular calcium dependent and could be mimicked by D-600, a known calcium entry blocker (Pang *et al.*, 1984).

2.2. The cardiac effect of PTH

Using isolated spontaneously beating rat right atria, Tenner *et al.* (1983) found that bPTH-(1-34) produced a concentration-dependent increase in beating rate. Prior to any significant chronotropic response, an increase in cAMP level occurred. Bogin *et al.* (1981) indicated that both bPTH-(1-34) and bPTH-(1-84) produced an immediate and sustained significant rise in beat frequency in isolated rat heart cells after 2 days of culture. They also reported that the PTH effect appeared to be due to the augmentation of calcium entry into heart cells. The evidence was as follows: 1) the calcium ionophore A23187 stimulated the heart cell beating rate in a manner that was similar to the stimulation produced by bPTH-(1-34); 2) the subsequent addition of bPTH-(1-84) to medium already containing verapamil, a blocker of calcium channels, did not produce stimulation of heart cells. When verapamil was added to PTH-stimulated heart cells, the beating rate declined within 1 min to the original baseline values. These observations are similar to those of Larno *et al.* (1980) who used rat myocardial cells cultured for 8 days. In addition to the vasodilation of canine vascular beds, PTH also induced a positive chronotropic effect in Nickols and Cline's studies (1983). In rabbit sinus node cells and guinea pig papillary muscles, Kondo *et al.* (1988) reported that bPTH-(1-34) had a positive inotropic effect, which was abolished by verapamil, a low calcium medium (0.12 mM), or methoxyverapamil (Katoh *et al.*, 1981). In voltage clamp experiments using the single sucrose-gap method, bPTH-(1-34) caused an increase in the peak amplitude of the slow inward current (+37%), while it did not affect the outward current (Kondo *et al.*, 1988). The interpretation of the above experiments was that PTH could act as a calcium channel activator in cardiac tissues. Two mechanisms may be responsible for this effect. First, the hormone may directly enhance calcium inward currents through voltage-dependent calcium channels. Second, PTH-stimulated cAMP production may indirectly modulate calcium channel currents.

2.3. The neuronal distribution and function of PTH

The effect of PTH on the calcium channels in neurones has not been investigated to date. The existence of PTH-like immunoreactivity in brains and other neural tissues of vertebrates has been demonstrated (Lack *et al.*, 1988; Pang *et al.*, 1988b, c). However, the role of PTH in the central nervous system (CNS) is not clear. One possible physiological function of PTH in the CNS might be to serve as a neurotransmitter or neuromodulator. By acting on specific PTH binding sites on the membrane of nerve cells, the peptide might regulate the entry of calcium by modulating the activities of second messengers or acting directly on calcium channels. Fraser *et al.* (1988a,b) showed that Na^+ gradient-stimulated Ca^{++} transport was significantly increased by 55% above control values in the presence of bPTH-(1-34), while bPTH-(3-34) itself had no effect. Although little information regarding the Na/Ca exchanger in nerve cells is available (Satin, 1984; Nachsen, 1985; DiPolo and Beauge, 1990), it has been suggested that the Na/Ca exchanger is not essential in buffering $[Ca^{++}]_i$ in normal or elevated resting $[Ca^{++}]_i$ in *Aplysia* neurons (Levy and Tillotson, 1988). Only under some pathophysiological conditions, does the role of the Na/Ca exchanger become important in some neurons (Fraser *et al.*, 1988). In light of these reports, the contribution of the Na/Ca exchanger to the PTH-mediated transmembrane movement of calcium appears to be small under physiological conditions (Somlyo, 1987).

In other excitable cells such as cardiac myocytes, the Na/Ca exchanger is involved in both calcium influx and efflux (Reuter and Seitz, 1968; Kalix, 1971; Blaustein and Oborn, 1975). In nonexcitable cells, the Na/Ca exchanger participates only in calcium efflux (Barritt, 1982). Calcium entry through Na/Ca exchanger in cardiac muscle did not affect significantly the rise rate of the normal $[Ca^{++}]_i$ transient (Crespo *et al.*, 1990); nor did calcium entry via the Na/Ca exchanger affect on calcium-induced calcium release from sarcoplasmic reticulum in cardiac cells under normal conditions (Lederer *et al.*, 1990). However, the cardiac Na/Ca exchanger is important in calcium extrusion during repolarization (Reeves and Hale, 1984; Lipp and Pott, 1988; Giles and Shimoni, 1989; Crespo *et al.*, 1990). The physiological importance of the Na/Ca exchanger in smooth muscle cells is controversial. Nabel *et al.* (1988) reported the existence of a bidirectional Na/Ca exchanger in cultured rat aortic vascular smooth muscle cells. These authors suggested that this Na/Ca exchanger seemed to play a role in Ca^{++} homeostasis, especially under conditions of altered intracellular Na^+ or increased $[Ca^{++}]_i$. At normal resting $[Ca^{++}]_i$ levels, the sarcolemmal Ca-pump may be more important in extruding Ca^{++} from smooth muscle cells. Since the Na/Ca exchanger is driven by two forces, namely $[Ca^{++}]_i$ and membrane potential (V_m), the increase in $[Ca^{++}]_i$ and membrane hyperpolarization will increase the net Ca^{++} extrusion by this co-transporter (Lauger, 1987; Nabel *et al.*, 1988). However, Na/Ca exchange seems to be insensitive to V_m in vascular smooth muscle cells. Hence, Nelson *et al.* (1990) speculated that the contribution of the Na/Ca exchanger to the overall voltage dependence of $[Ca^{++}]_i$ in vascular smooth muscle cells is very small.

In summary, PTH may play an important role in the physiological regulation of transmembrane movement of calcium. In vascular smooth muscle cells, PTH can inhibit calcium entry upon depolarization. In cardiac cells, PTH has positive chronotropic and inotropic effects which could be due to the increased slow inward calcium current. In neuronal cells, PTH is also involved in the mediation of Ca^{++} transport. All the evidence accumulated thus far suggests that PTH might be an endogenous calcium channel antagonist in some tissues such as vascular smooth muscle, or an endogenous calcium channel agonist in other tissues such as cardiac muscle. By having different effects on calcium channels in various tissues, PTH may be able to regulate excitation-contraction and stimulation-secretion couplings and coordinate the activities of different organ systems in our body. However, this new hypothesis which envisions PTH as an endogenous peptidergic calcium channel modulator needs direct electrophysiological evidence.

3. The role of cyclic AMP in the modulation of voltage-dependent calcium channels and the cardiovascular effect of PTH.

Voltage- and time-dependent calcium channels which are regulated directly by the binding of a transmitter or a hormone to the channel protein have thus far not been identified. However, a large body of evidence indicates that the hormonal modulation of calcium channels is mediated by some second messengers, such as cAMP and cGMP. Three distinct classes of protein kinases have been shown to regulate Ca^{++} currents in excitable tissues. They are cAMP-dependent protein kinase (Tsien, 1983; Sperelakis and Wahler, 1988), cGMP-dependent protein kinase (Martins *et al.*, 1982; Wahler *et al.*, 1990), and Ca^{++}/diacylglycerol-dependent protein kinase (protein kinase C) (Galizzi *et al.*, 1987; Fish *et al.*, 1988). Furthermore, the direct action of G proteins on calcium channels has been demonstrated in more and more cell preparations (Yatani *et al.*, 1987a, 1988; Loirand *et al.*, 1990). In this brief review, the focus will be on the mediation of calcium channels by the cAMP pathway in neurons, smooth muscle cells and ventricular myocytes.

3.1. The modulation of calcium channel currents by cAMP in neurons, vascular smooth muscle and myocardial cells.

When Ca^{++} currents are measured during internal perfusion of d.r.g. neurons, the currents decrease with time. This "run-down" of calcium channel currents can be prevented by including cAMP in the perfusate (Fedulova *et al.*, 1981), suggesting the existence of a cAMP-dependent modulation of calcium channels. In addition, cAMP-dependent phosphorylation of calcium channel proteins has been demonstrated directly in skeletal muscle (Curtis and Catterall, 1985).

Intracellular perfusion of cAMP directly enhanced Ca^{++} current in rat d.r.g. neurons (Fedulova *et al.*, 1985). Narahashi *et al.* (1987) reported that cAMP alone increased L channel currents and decreased T channel currents in N1E-115 cells. The increase in calcium channel currents induced by NA in hippocampal granule neurons (Gray and Johnston, 1987) was also proposed to be regulated by cAMP because the direct application of a membrane-permeable analogue of cAMP or forskolin mimicked the action of NA. cAMP-mediated phosphorylation of calcium channels was also suggested to be involved in the stimulatory action of CGRP on Ca^{++} currents in rat d.r.g. neurons (Ryu *et al.*, 1988). Furthermore, serotonin-induced enhancement of Ca^{++} currents (Kramer *et al.*, 1988) seems to result from an increase in cAMP (Levitan and Levitan, 1988), which presumably activates calcium channel proteins (Levitan, 1985).

A rise in cAMP concentration causes relaxation of many types of smooth muscle including vascular smooth muscle. Four mechanisms for this action have been postulated, none of which excludes the others. cAMP may decrease the Ca^{++} available to the contractile proteins through: 1) increased Ca^{++} sequestration into intracellular storage sites (Mueller and van Breemen, 1979); 2) decreased Ca^{++} influx (Meisheri and van Breemen, 1982; Abe and Karaki, 1988, 1989); 3) increased Ca^{++} efflux (Scheid and Fay, 1984); or 4) inhibited myosin light chain kinase activity (Adelstein *et al.*, 1978; Conti and Adelstein, 1980). The stimulation of β-adrenoceptors can increase the cellular content of cAMP in smooth muscle cells (Bülbring and Tomita, 1969). Further studies by Meisheri and van Breemen (1982) indicated that isoproterenol and db-cAMP inhibited the ^{45}Ca influx elicited by K-depolarization in whole smooth muscle tissues of rabbit aorta. Using the patch clamp technique, Droogmans *et al.* (1987) reported that 10 μM isoproterenol inhibited L channel currents in single vascular smooth muscle cells from the rabbit ear artery. In addition, the presence of cAMP in the pipette solution was necessary to inhibit the "run down" of inward currents of calcium channels in rat d.r.g. neurones (Fedulova *et al.*, 1985), while this was not the case in smooth muscle cells (Klöckner and Isenberg, 1985b). However, some studies have suggested that calcium channels in smooth muscle might not be controlled by cAMP (Ohya *et al.*, 1987a; Tomita, 1988). A direct effect of cAMP on voltage-dependent calcium channels, especially in vascular smooth muscle cells, has not been completely and convincingly determined. This uncertainty is due to many factors, such as the cell preparation, the concentration of cAMP and the target protein kinases of cAMP. For example, both cAMP- and cGMP-dependent protein kinases can be activated by cAMP (Lincoln *et al.*, 1990). The activation of the former may result in an increase in L channel currents while the activation of the latter may lead to the inhibition of L channel currents. In primary cultured vascular smooth muscle cells, both of these two protein kinases exist. However, only cAMP-dependent protein kinase could be detected in long-term cultured vascular smooth muscle cells (Lincoln *et al.*, 1990).

Actions of cAMP on cardiac muscle seem to differ from those observed with smooth muscle (Ohya *et al.*, 1987a). The Ca^{++} current is increased by activation of the cAMP-dependent phosphorylation of the Ca^{++} channel or a closely associated regulatory protein in cardiac myocytes (Osterrieder *et al.*, 1982; Wahler *et al.*, 1990). Butyryl derivatives of cAMP increased the slow inward current in cardiac Purkinje fibres (Tsien *et al.*, 1972), and dibutyryl cAMP enhanced fluxes of $^{45}Ca^{++}$ into heart cells (Meinertz *et al.*, 1973). It is known that the activation of β-adrenergic receptors by β-agonists leads to dissociation of G_s into α_s and βγ-subunits. The α_s-subunit combined with GTP and, consequently, activates the catalytic subunit of adenylate cyclase. The subsequently increased intracellular cAMP content results in an increase in Ca^{++} current in cardiac muscle cells. However, the increase in cAMP content cannot explain all β-adrenergic effect on cardiac calcium channels. In single channel studies, for example, with cardiac myocytes from cow, cat and guinea pig, injection of cAMP did not affect the amplitude of the single channel currents or the total number of channels. However, the average currents were increased by a factor of 1.4 due to the increase in the probability of the channel being open during depolarization (Brum *et al.*, 1984). The voltage dependence of β-adrenergic actions on cardiac calcium channels has also be reported. Isoproterenol increased Ca^{++} currents at +30 mV by two-fold, but did not significantly increase Ca^{++} currents at -20 or -10 mV (Bean 1985). In this regard, the direct effect of G_s on cardiac calcium channels is suggested. Yatani *et al.* (1987a) observed the activation of cardiac Ca^{++} channels by the addition of preactivated G_s or its α subunit, using membrane patches from guinea pig myocytes. After incorporation into the phospholipid bilayer, the α subunit of G_s directly activated cardiac Ca^{++} channels (Imoto, *et al.*, 1988). Thus, the effect of β-adrenergic agonists on cardiac Ca^{++} channels is suggested to be mediated by two mechanisms, a cAMP-independent, membrane-confined mechanism and a cAMP-dependent cytosolic component-required mechanism. Both of mechanisms require the activation of G_s (Schultz *et al.*, 1990).

On the other hand, acetylcholine (Ach) and other muscarinic agonists decrease the Ca^{++} current in cardiac myocytes. The direct inhibition of adenylate cyclase activity via the Ach-induced activation of inhibitory G proteins (G_i and/or G_o) (Hescheler *et al.*, 1986; Nakajima *et al.*, 1990) has been postulated. The mediation by the cGMP pathway is another one of possible mechanisms for the muscarinic effects on cardiac Ca^{++} currents (see review by Tsien, 1983; Fischmeister and Hartzell, 1987; Sperelakis and Wahler, 1988).

3.2. The cAMP mediation of the PTH-induced cardiovascular effect.

Pang *et al.* (1984) reported that PTH simultaneously inhibited Ca^{++} entry and increased cAMP concentration in vascular smooth muscle cells. In both *in vivo* and *in vitro* studies, phosphodiesterase inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) potentiated, and phosphodiesterase simulators such as imidazole inhibited, the vascular action of bPTH-(1-34). More direct evidence came from the determination of the cAMP concentration in rat tail artery, which increased in the presence of bPTH-(1-34) in a time-dependent fashion. In addition, PTH relaxed rabbit aortic strips and ring segments, and increased intracellular cAMP concentrations in cultured rat and rabbit aortic smooth muscle cells. This evidence suggested a mediation of the vasodilatory action of PTH by cAMP (Nickols, 1985). Huang *et al.* (1983, 1984) also reported stimulation of cerebral artery membrane adenylate cyclase by bPTH. In isolated perfused rat mesenteric vascular beds, the cyclicnucleotide phosphodiesterase inhibitor, methylisobutylxanthine, did not reduce the contraction of the blood vessels by itself, but significantly enhanced the vasodilatory action of bPTH-(1-34) (Nickols *et al.*, 1986). It would appear that PTH-stimulated cAMP production is related to the inhibition of vascular smooth muscle cell contraction, or that these two events are temporally coupled. Unfortunately, all these reports did not reveal whether increased cAMP itself would modulate the activity of calcium channels.

There was a marked and significant increment in cAMP production in primary cultured rat heart cells within 30 sec of the addition of PTH to the medium. Almost at the same time, the slow inward calcium current (Kondo *et al.*, 1988) and the beating rate (Bogin *et al.*, 1981) were increased. Thus PTH elevates cAMP levels in both vascular smooth muscle cells and cardiac cells. How can this peptide block calcium channels in smooth muscle cells and increase calcium channel currents in cardiac cells? The answer may, in part, be that PTH exerts its influences on calcium channels through different pathways in various cell types.

4. Objectives

The hypothesis that forms the basis of this investigation is that PTH modulates calcium channel currents. There are three types of evidence to support this hypothesis. They are: 1) the dependence of the PTH effect on extracellular calcium; 2) the modulation of $^{45}Ca^{++}$ uptake by PTH during KCl challenge; and 3) the modulation by PTH of inward currents via calcium channels in dispersed single cells. The first two points have been firmly established by Pang and his co-workers (1984, 1985a, 1988a). Their data suggest that PTH modulates calcium channels. This hypothesis will be further explored in the present study.

4.1. Characterization of voltage-dependent calcium channel currents in

neuroblastoma cells, vascular smooth muscle cells and ventricular myocytes.

The existence of calcium channels in rat tail artery smooth muscle cells has not previously been demonstrated using electrophysiological techniques. This was due to problems in the isolation of cells and the recording of currents (Toro *et al.*, 1986). Although several groups have reported the existence of T and L channels in other vascular smooth muscle cell preparations, the first characterization of the T and L channel currents in rat tail artery smooth muscle cells was by Wang *et al.* (1989) (see Chapter 4). In neonatal rat ventricular myocytes, T and L channel currents were not fully characterized. In addition, the separation of T from L channel currents in neuroblastoma cells was not satisfactory due to overlap of the steady-state inactivation potentials (Narahashi *et al.*, 1987). Hence, experiments were carried out to characterize further the T and L channel currents in these three cell preparations.

4.2. Investigation of the effects of PTH on voltage-dependent calcium channel currents in neuroblastoma cells, vascular smooth muscle cells and ventricular myocytes.

The cardiovascular effects of PTH are dependent on extracellular calcium and can be blocked by calcium channel antagonists. PTH also affects the intracellular calcium level in neurons. However, there are no reports concerning the PTH effect on different voltage-dependent calcium channels. Furthermore, it is very important to determine whether PTH has the same effect on calcium channels in different cell preparations, such as cardiac cells and vascular smooth muscle cells. The results will aid our understanding of the tissue specificity of PTH effects.

4.3. Delineation of the mechanisms of PTH effects on voltage-dependent calcium channel currents in vascular smooth muscle cells and ventricular myocytes, especially the role of cAMP in the modulation of PTH effects on voltage-dependent calcium channel currents.

A temporal coincidence of cAMP production and cardiovascular responses in the presence of PTH has been demonstrated. The application of cAMP mimicked PTH-induced vasoconstriction and its positive inotropic effect. However, it is not known if the PTH-elicited cAMP production is directly related to the cardiovascular responses to PTH. Furthermore, increased cAMP content enhances calcium channel currents in myocardial cells but decreases the stimulated elevated level of intracellular calcium in vascular smooth muscle cells. Hence, the present study is also aimed at determining whether cAMP is the second messenger which mediates the PTH-induced tissue selective effect on L channels.

CHAPTER 2

Methods

1. Cell preparation

1.1. Neuroblastoma cells (N1E-115).

Cells of the mouse neuroblastoma clone N1E-115 were cultured at 37^{0}C in a humidified atmosphere of 5% CO_2 in room air. The Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS, Gibco) was changed every 3 or 4 days and the cells used before they became confluent. After mechanical agitation, approximately $3x10^4$ cells were replated per 35 mm tissue culture dish. Four to six hours after replating, a Petri dish with attached cells in 3 ml bath solution was mounted on the stage of an inverted phase contrast microscope for recording calcium channel currents (Kimhi *et al.*, 1976). N1E-115 cells which were cultured as described above were spheroidal in shape and had no visible neural outgrowths. The mean diameter of these cells was approximately 25 µm. Predominately T-channel currents were expressed in these cells.

In other experiments, the cells were grown and maintained at confluence for 3 to 4 weeks under the same culture conditions, but in addition, 2% DMSO was included in the medium. Three to five days before use, the cells were replated in a culture dish and maintained in medium containing 2% DMSO (Moolenaar and Spector, 1978). These cells differentiated more rapidly and usually had one to three short neurites. Predominately L channels were detected in these cells.

1.2. Vascular smooth muscle cells from rat tail artery

Male Sprague Dawley rats (100-200 g body wt) were anaesthetized with sodium pentobarbital (65 mg/kg wt ip), and the tail artery was dissected out and immersed in cold low calcium (0.2 mM) Hank's Buffered Saline Solution (HBSS, Gibco). The blood was washed off the artery, and the connective tissue was then removed. With the use of a dissecting microscope inside a laminar flow hood, the artery was cut open longitudinally and placed in 4°C low calcium HBSS for 30 min. After this incubation, the medium was changed to *enzyme solution I*, which was composed of collagenase/dispase (1.5 mg/ml, Boehringer Mannheim GmbH), elastase (0.5 mg/ml, Sigma type II-a), trypsin inhibitor (1 mg/ml, Sigma type I-s) and bovine serum albumin (2 mg/ml, fatty acid-free, Sigma). The tissue was incubated in this solution for 60 min. The medium was then changed to *enzyme solution II* which was composed of collagenase (1 mg/ml, Sigma type II), trypsin inhibitor (0.3 mg/ml, Sigma type I-S) and bovine serum albumin (2 mg/ml, Sigma). Incubation in enzyme solution II also lasted 60 min. All incubations in enzyme solutions were carried out in a CO_2 incubator (5% CO_2 and 95% room air at 37°C). Usually two tail arteries were treated simultaneously in 5 ml of enzyme solution.

The arteries were then triturated using a fire polished Pasteur pipette until the medium turned cloudy. The cell suspension was stored in the refrigerator at 4°C and the Ca^{++} concentration of the suspension was increased in a stepwise fashion over a 40 min period to 2.0 mM, after which the cells were placed in 35 mm culture dishes in DMEM. To facilitate the attachment of the cells, FCS was excluded from the medium (DMEM) for the first 4-6 hr of the culture. After this initial period, 10% FCS was added to the medium. The cells were cultured in a CO_2 incubator at 37°C and were used within 8-36 hr after they were plated. The viability of the cells was tested using trypan blue exclusion (Bagby *et al.*, 1971; Ives *et al.*, 1978). In our preparations more than 95% of the cells did not take up the dye. These cells contracted in the presence of norepinephrine (10 μM) as observed under phase contrast optics (Nikon, Japan) (Marvin *et al.*, 1979; Nakazawa *et al.*, 1987).

1.3. Ventricular myocytes from neonatal rat

Neonatal rats (3 days old) were used in the present study. After decapitation, the top 1/3 of the rat heart was discarded and the ventricles were removed aseptically and incubated in Ca^{++}-free HBSS for 1 hr at 4°C, and then minced under a dissection microscope. Thereafter, two enzymatic methods were used to disperse single ventricular myocytes. The quantity and quality of harvested myocytes from these two methods were very similar.

Method I: The tissue was incubated at 37°C for 10 min with Ca^{++}-free HBSS containing trypsin (0.5 mg/ml, I-300, United States Biochemical Co.). The tissue was then exposed to fresh trypsin solution again for 10 min and was shaken continuously. Next, the tissue was digested with another enzyme solution containing collagenase/dispase (1.5 mg/ml, Boehringer Mannheim Gmbh Co.), elastase (0.5 mg/ml, Sigma type II-a), trypsin inhibitor (1 mg/ml, Sigma type I-s) and bovine serum albumin (2 mg/ml, fatty acid-free, Sigma) for 30 min at 37°C. The solution was also gently agitated. The cell suspension was centrifuged at 1000 rpm for 5 min, and the supernatants discarded.

Method II: The tissue was incubated at 37°C for 20 min with Ca^{++}-free HBSS containing trypsin (2 mg/ml, type-II, Sigma) and bovine serum albumin (2 mg/ml, fatty acid-free, Sigma). Then the plaque of tissue was exposed to the fresh trypsin solution again for 15 min at a constant rotating speed. At the end of this digestion period, the supernatant was collected and diluted in DMEM + 10% FCS, and stored at +4°C. The remaining tissue pellet went into the next trypsin digestion. This process was repeated four to five times. Finally, all of the cell suspensions collected from above digestion steps were centrifuged at 1000 rpm for 5 min, and the supernatants discarded.

The remaining tissue fragments were triturated for 3 min and then filtered through a nylon mesh with a pore size of 250 μm and centrifuged again. The filtrate was cultured at 37°C for two hours to exclude non-muscle cells (Polinger, 1970). The unattached cells were then seeded in culture dishes and grown in DMEM supplemented with 10% FCS. These cells were used within 24 hours of dispersion. The harvested myocytes grew into a confluent beating monolayer after a 24 hr incubation (37°C, 5% CO_2, 95% humidified air). The voltage clamp studies were carried out before the cell monolayer was formed. All the patch clamp experiments were carried out on myocytes which were spherical or elliptical in shape.

2. Patch clamp technique

2.1. Solutions.

The bath (extracellular) solution contained (mM): Tris,110; CsCl,5; HEPES,20; glucose,30; $BaCl_2$,20; KCl,5 and TTX,0.5 μM. In all experiments, Ba^{++} was used instead of Ca^{++}. Incorporation of Tris in the bath solution caused less leakage and better survival of the cells (Moolenaar and Spector, 1978). The Na^+-free bath solution containing 20 mM Ba^{++} abolished inward currents carried by sodium ions. It is also possible that some permeable ions other than Na^+ might go through the Na^+ channels. The addition of 0.5 μM tetrodotoxin (TTX) to the bath solution to effectively block Na^+ channels eliminated this possibility (Hagiwara and Nakajima, 1965). Although Na^+ currents were absent in the majority of vascular smooth muscle cells (Toro *et al.*, 1986; Tomita, 1988), the use of TTX in the Na^+-free bath solution effectively eliminated any inward current through sodium channels.

In order to eliminate outward K^+ currents and to avoid Ca^{++}-dependent inactivation of the inward Ca^{++} currents, 20 mM Ba^{++} was used as the charge carrier for the inward current. Ba^{++} is more permeable than Ca^{++} through L-type calcium channels. Although Meech and Standen (1975) reported that barium was effective in increasing the potassium conductance, most of the recent studies have demonstrated that a high concentration of Ba^{++} in the external solution blocked the Ca^{++}-activated K^{+} channel (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Benham *et al.*, 1985). Furthermore, either zero or 10 mM K^{+} in the pipette solution makes the contaminating K^{+} currents negligible. In addition, a high concentration of Cs^{+} in the pipette solution blocks K channels (Klöckner and Isenberg, 1985b). The influx of Ba^{++} during repeated depolarization has been reported to have no significant inhibitory effect on the Ca^{++} channel (Brown *et al.*, 1981; Lee *et al.*, 1985; Ohya *et al.*, 1988).

Two kinds of pipette solutions were used in the study. The pipette (intracellular) solution used for neuroblastoma cells was composed of CsCl 130 mM, HEPES 20 mM, glucose 5 mM, EGTA 4 mM, $MgCl_2$ 5 mM, ATP 2 mM and cAMP 0.5 mM. Cesium is known to be able to block the outward K^{+} current (Quandt and Narahashi, 1984), and the addition of ATP and cAMP to the pipette solution with Mg^{++} delays the run-down of the calcium channels (Fedulova *et al.*, 1985).

The pipette solution used for vascular smooth muscle cells and ventricular cells contained (mM): Cs_2-aspartate 75, EGTA 10, ATP 2, $MgCl_2$ 5, K-pyruvate 5, K-succinate 5, glucose 25, HEPES 15, creatinphosphate-Na_2 5, and creatin-kinase, 50 units/ml (Boehringer Mannheim Gmbh Co.). The advantage of the composition of this solution will be discussed in Chapter 4.

Since 10 mM EGTA was included in the Ca^{++}-free pipette solution, the concentration of intracellular free calcium was estimated to be less than 10^{-9} M (Hille, 1974; Hagiwara and Byerly, 1981; Stockbridge, 1987; Sada *et al.*, 1988). The low concentration of intracellular calcium will help to decrease the calcium-induced inactivation of calcium channel currents. However, the activity of some intracellular calcium dependent enzymes including cAMP-dependent protein kinase, will be also decreased, and in turn, the hormonal effects mediated by these enzymes may also be decreased in the presence of low intracellular calcium levels.

Before use, all solutions were filtered through a filter with a pore size of 0.22 µm. The pH of both bath and pipette solutions was adjusted to 7.4 with HCl for the bath, and CsOH for the pipette, solutions, respectively. The osmolality of both solutions was adjusted to 320 mOsm.

In order to check the effectiveness of the inhibition of outward K^+ channel currents in some experiments, 20 mM TEA was also included in the bath solution. TEA blocks delayed rectifier and Ca^{++}-activated K^+ channels (Moolenaar and Spector, 1978; Fishman and Spector, 1981; Hugues *et al.*, 1982). There was no measurable change in the inward currents recorded with TEA in the solution. At the end of most experiments, 2 mM La^{+++} was added to the bath solution and the recorded inward currents were completely eliminated. In addition, 4 mM EGTA, which was included in the bath solution in some cases, also eliminated the recorded inward currents. Finally, in some experiments high concentrations of Ba^{++} (50 mM) or different concentrations of Ca^{++} (2.5 or 20 mM) were used in Na^{+} free bath solutions. Under these conditions, the inward current amplitudes were increased or decreased and the apparent reversal potentials varied, correspondingly. The above manipulations insured that the inward currents recorded in the present study reflected calcium channel activity.

2.2. Whole-cell recording

2.2.1. Recording and off-line analysis of current records

In experiments with neuroblastoma cells, the recording and analysis of current records was accomplished using a digital oscilloscope (Nicolet Co., Model 206) and its associated disk drive. Test pulses were generated with the use of a digital stimulator.

In experiments using vascular smooth muscle cells and ventricular myocytes, the current traces were digitized using a personal computer system. Two software packages were used to analyze data. 1). VCAN, version 1.2. (software package provided by Dr. J. Dempster, University of Strathclyde, Glasgow) was used to drive the DT2801A interface (Data Translation, Inc.). 2). pClamp, version 5.5.(software package purchased from Axon Instruments, Inc.) was used to drive the TL-1 DMA interface (Axon Instruments, Inc.). Depolarization pulses were generated by a personal computer through a digital-to-analog converter.

2.2.2. Procedures of current recording.

Pipette: The pipette for recordings in neuroblastoma cells was made of borosilicate thick wall glass capillary tubes (OD=1.2 mm, ID=0.6 mm) and had a resistance of 8-10 MΩ. The pipette for recordings in vascular smooth muscle cells and ventricular myocytes was made of borosilicate thin wall glass capillary tubes (OD=1.2 mm, ID=0.9 mm) and had a resistance of 2-8 MΩ. These pipettes were pulled by a two stage electrode puller (Narishige, PP-83, Japan) and firepolished using a microforge (Leitz, Wetzlar).

Space clamp: The diameter of neuroblastoma cells cultured in the medium without DMSO was approximately 25 μm. After culture in medium with 2% DMSO for one month, the cells sprouted several short neurites. In order to obtain a good space clamp, cells with a diameter approximately 36 μm and a neurite length less than the soma diameter were chosen (Tsunno *et al.*, 1986). After the culture medium was replaced by the recording bath solution with 20 mM Ba^{++}, almost all of the primary cultured vascular smooth muscle cells and ventricular myocytes were spherical in shape with a diameter of approximately 20 - 40 μm. An optimal space clamp was, therefore, usually obtained. An acceptable temporal and spatial clamp was evident from the following observations. 1) The capacitive current settled within 2-6 msec, fast enough to separate it from the slow inward calcium currents. Furthermore, the capacitive current recorded under patch clamp conditions had an exponential decline. 2) The activation, inactivation (T-type), and slow inactivation (L-type) segments of inward current at a fixed test pulse did not show any abnormal notches which was a sign of a poor spatial clamp. 3) The current vs voltage (I-V) relationships of predominantly T or L channel currents were smooth and bell-shaped. No abrupt changes of inward current corresponding to the direction of either hyperpolarization or depolarization were observed (Colatsky *et al.*, 1979).

There was a slow contraction observed during the sequential depolarizing stimulation of the vascular smooth muscle cell. During that time, the cell became smaller and more spherical. This progressive cell contraction did not pull the cell away from the pipette or the bottom of the Petri dish, and the membrane current was not significantly altered as demonstrated by stable recording over a 20 min period.

Compensation of leakage current, capacitive transient and series resistance: At the beginning of the experiment, the junction potential between the pipette and the bath solution was zeroed by adjusting the pipette current to zero. In the presence of 2 mM La^{+++}, the current vs voltage (I-V) relationship of the leakage current is linear, for which the intracellular Cs^+ may be partially responsible. Linear leakage and capacitive currents were subtracted with the use of Axopatch-1B electronics, or software (VCAN or pClamp), or by using currents elicited from a small hyperpolarizing pulse at the beginning and the end of the experiment. In most of the experiments described in this thesis, unless otherwise indicated, pClamp was used to subtract leakage and capacitive currents.

The accuracy of the voltage clamp over the whole cell membrane is affected by the product of the magnitudes of series resistance and of the membrane currents. In case of a large series resistance, a deviation of the membrane potential from the command voltage is present. In neuroblastoma and vascular smooth muscle cells, series resistance compensation was not employed because the peak inward currents measured with 20 mM Ba^{++} as the charge carrier were usually small (less or about 200 pA) and the series resistance was usually less than 10 megohms (estimated using Axopatch compensation). The voltage error due to series resistance was, hence, less than 2 mV. In cases where the voltage error was greater than 2 mV, especially in N1E-115 cells, series resistance compensation was used (Hodgkin and Huxley, 1952a). In ventricular myocytes, the optimum compensation of series resistance was made electronically, using the Axopatch-1B. If the compensation was not satisfactory, the data from the cell were not used.

Seal and penetration: The standard gigaohm seal, whole-cell version of the patch clamp technique was used to measure whole-cell inward currents (Hamill *et al.* 1981). A Petri dish with attached cells was mounted on the stage of an inverted phase contrast microscope (Nikon, Japan) and monitored using a video camera (JVC,GX-S 700). Currents were filtered with a 4-pole Bessel filter at a cutoff frequency of 5 KHz. The pipette, which had a small positive internal pressure in order to keep the tip clean, was pushed onto the cell surface by using a three dimensional micromanipulator (Narishige, Japan). A tight seal (gigaseal) between the membrane and the tip of the pipette (10 to 50 gigaohm) was obtained by the application of suction through the pipette. Further suction disrupted the membrane under the tip of the pipette, and then the pipette solution dialysed the cell. During the process of forming a gigaohm seal between the tip of the pipette and the membrane patch, some vascular smooth muscle cells or ventricular myocytes contracted to such a degree that a gigaohm seal could not be achieved. These cells could not be used. The experiments were carried out at room temperature (20-22°C) in order to insure a longer survival time of impaled cells and a better time resolution of the fast membrane currents (Moolenaar and Spector, 1978). The currents were amplified by an Axopatch-1B (Axon Instrument, Inc., USA) patch-clamp amplifier, or by a List EPC-7 (List-Electronic, FRC) amplifier, with a 0.5 gigaohm feedback resister. In most cases, pulses of 200 msec duration were applied at intervals of 5 to 10 sec to allow complete recovery of calcium channel activity after inactivation.

Run-down: The decline of calcium channel currents with time ("run-down") is a universal phenomenon in patch-clamp studies. In the present study, much effort was devoted to exclude the interference of "run-down" of calcium channel currents. In vascular smooth muscle cells and neuroblastoma cells, immediately after the cell was voltage clamped, the inward current increased, possibly as the result of the outward K^+ current being blocked by a high concentration of Cs^+ in the pipette and Ba^{++} in the bath (Armstrong and Taylor, 1980; Quandt and Narahashi, 1984). This transient increase in the amplitude of calcium channel currents was absent in ventricular myocytes. A relatively stable period of calcium channel currents was observed in a latter phase in all three cell preparations. If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of "run-down" of the inward current was usually negligible for 20 min or longer (Wang *et al.* 1989; Pang *et al.*, 1990). Cells which had stable inward currents from the third to fifth minute after penetration of the membrane were used. If the current decayed too quickly, it was difficult to distinguish "run-down" of the current from the inhibitory or excitatory effect of the agents. Cells which had a fast decline of inward currents within this period were discarded.

At the end of the fifth minute, peak currents of calcium channels were recorded and the I-V relationship was plotted as the control. Different agents were then added directly to the bath solution and the change in the inward current was monitored continuously. One to three min were required for the agents to be distributed evenly throughout the bath solution. Hence, I-V relationships of calcium channels were obtained after at least 3 min exposure of the cell to each agent to obtain a stable response of the calcium channel currents. When the recorded amplitude of the inward currents was 5% higher or lower than that obtained at the previous recording (30 second interval), an increase or decrease, respectively, in the amplitude of the inward current was recognized. In some cells the bath solution was changed to wash out the tested agent in order to determine whether the effects of these agents are reversible. This procedure further excluded the influence of "run-down" of calcium channel currents. No allowance, hence, was made for the spontaneous decline of the inward current in measuring the effect of various agents on the calcium channel current. In an attempt to investigate the specificity of the effect of an agent on L channel currents, a dihydropyridine antagonist or an agonist was, in some cases, applied before or after the application of the agent to the same cell.

Current Fitting: In vascular smooth muscle cells, it is difficult to fit the kinetics of L channel currents exponentially. Hence, in some cases, the half times ($T_{1/2}$) of inactivation and activation of L channel currents were used (Bean, 1985; Cavalie *et al.*, 1985; Malecot, 1988). The half times of activation and inactivation are the times at which L channel currents reach 50% or decline to 50% of the peak current amplitude.

In atrial cells, the inactivation of T, but not L, channel currents was fitted with a least-squares single exponential equation (Bean, 1985). L channel currents in guinea-pig ventricular cells showed a two exponential time course of inactivation (Markwardt and Nilius, 1990). However, Ochi and Kawashiwa (1990) reported that the mean currents of calcium channels in guinea-pig ventricular cells decayed with a single-exponential time course. Cohen *et al.* (1987, 1988) were also able to fit the L channel currents in rat ventricular myocytes to a single exponential equation. In the present study, L channel current traces in neonatal rat ventricular myocytes were fitted well in a bi-exponential mode with a least squares residual (R) larger than 0.9, generating, in most cases, single time constants for activation and inactivation, respectively. The fitting procedure produces the best fit by using algorithms that minimize the least squares error between data points and calculated fit points. If a satisfactory fit could not be achieved, i.e.,either an R value less than 0.8 or termination of the fitting procedure by the computer indicating the data being out of the fitting range, the data was not used.

2.2.3. The separation of T channel currents from L channel currents

Three methods were used to separate T channel currents from L channel currents, i.e. culture method, electrophysiological and pharmacological separation.

Culture method: In neuroblastoma cells, after culturing cells in the medium with 2% DMSO for one month, L channel currents became the predominant component and T channel currents were almost completely eliminated.

Electrophysiological method: Two procedures were used to separate T and L channel currents. The peak amplitude of the inward current evoked from a holding potential of -80 mV was taken as the sum of the T and L channel currents or total currents. The amplitude of the L channel current was detected at the end of a 200 msec test pulse and the T current was determined by subtraction of the L channel current from the total currents. Otherwise, subtraction of the amplitude of L channel currents recorded at a holding potential of -40 mV from the amplitude of total currents recorded at a holding potential of -80 mV was used to obtain the amplitude of T channel currents. The results from these two methods are similar.

Pharmacological method: A variety of synthetic compounds have been classified as calcium antagonists and agonists (Fig. II-1). Some of them have been widely used clinically to treat certain cardiovascular and brain disorders (Olivari *et al.*, 1979; Kimura and Kishida, 1981; Overweg *et al.*, 1984). Different types of calcium channels have different pharmacological sensitivities. It is known that phenylalkylamines (e.g. D-600) and dihydropyridines (e.g. nifedipine) selectively block slow calcium channels in myocardium and skeletal muscle (Tsien *et al.*, 1987), while diphenylpiperazines (e.g. flunarizine) have no detectable action on slow calcium channels in myocardium (Godfraind, 1987). There are few studies on the selective inhibition of T channel currents by synthetic calcium channel blockers. In the present study, dihydropyridines are used to inhibit or increase L channel currents, respectively. D-600 is also used to examine the sensitivities of T and L channel currents in neuroblastoma cells.

2.2.4. Temperature control

After dissociation, the vascular smooth muscle cells were replated onto 25 mm cover slips. These cells were studied 8 to 36 hrs after replating. The cover slip with the attached cells was fitted into a thermally controlled chamber. The temperature of the extracellular solution in the chamber was maintained using a temperature controller (TC-102 Medical Systems Corp.). This controller maintains temperature in the range of 30° to 45°C ± 0.2°C. At temperatures below 30°C, the temperature of the extracellular solution was adjusted by electrically heating or cooling the chamber and measuring the temperature using a thermistor placed near the centre of the chamber. The temperature coefficient, Q_{10}, was used to measure the temperature sensitivity of calcium channels (Kimura and Meves, 1979):

$$Q_{10} = (X_2/X_1)^{10/(T_2-T_1)}$$

where X_1 is the value of the relative amplitude of calcium channel currents at the lower absolute temperature T_1 and X_2 is that at the higher absolute temperature T_2.

3. Morphological studies

Immediately after isolation, single vascular smooth muscle cells from rat tail arteries and single ventricular myocytes from neonatal rat ventricles were seeded on glass coverslips (22 mm round, Fisher). These cells were cultured in DMEM + 10% FCS at 37^{0}C in humidified air with 5% CO_2. Ten to twelve hours prior to the experiments, the DMEM + 10% FCS was replaced by fresh DMEM without FCS. The contraction of vascular smooth muscle cells and ventricular myocytes were monitored using a phase contrast microscope (Nikon, Japan) or the monitor screen associated with a video camera (JVC, GX-S 700). Furthermore, the morphological changes in the cells during the experiment were photographed at defined time intervals and taped continuously through a video camera recorder (Philips, HQ$^+$). Since the shape of vascular smooth muscle cells and ventricular myocytes are irregular, spheroidal, oval or elongated, the photographic area of the cells was used as the parameter to evaluate the contraction of these cells. The primary aim of the morphological studies was to determine the PTH effects in the absence of cell dialysis. In regard to this purpose, electrical measurements (current clamp or voltage clamp) were not carried out in the cells used for morphological observations. Consequently, cell capacitance was not used to measure cell surface area. The photographic area of a single cell was estimated by digitizing the cell outline on the photograph using a digitizing tablet and Sigmascan software (Jandel, USA). Cell contraction causes a significant reduction in muscle cell size.

After the Petri dish was removed from the incubator, the culture medium was aspirated and the cells were rinsed twice with recording solutions. The coverslip with attached cells was then mounted on the stage of the microscope. The cell shape was monitored continuously for 10 to 20 min until the temperature of the culture medium was equal to room temperature (20^0 to 24^0C). This was the temperature at which all the measurements were carried out.

3.1. Experimental protocols for smooth muscle cells

After the areas of single smooth muscle cells in the culture medium were recorded, modified Tyrode solution was used to perfuse cells. The composition of the modified Tyrode solution is as follows: 90 mM NaCl, 60 mM KCl, 3.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES. The pH of this solution was adjusted to 7.4 using NaOH and the osmolality was adjusted to 320 mOsm. The area of the cells was monitored continuously for 10 min after cell contraction was induced by the KCl challenge. In another series of experiments, 1 μM bPTH-(1-34) was added to the modified Tyrode solution to perfuse the cells. The changes in the cell area were observed for the ensuing 15 min.

3.2. Experimental protocols for ventricular myocytes

During the experiment, FCS-free DMEM was used as the bath solution. The quiescent myocytes were directly exposed to 1 μM bPTH-(1-34). The changes in the cell area in the absence and then presence of bPTH-(1-34) were recorded. In some cases, at the end of the experiment, nifedipine was added to the same dish.

When the spontaneous beating of single ventricular cells was being measured, ventricular cells were used 18 to 40 hrs after being cultured. The beating of ventricular myocytes was observed under low power (40x or 60x) phase contrast microscopy and counted with a stopwatch either on site or on the monitor screen by replaying the video tape. The beating rate (times/min) was taken as a mean value of 3 separate measurements. Control beating was normalized as 100%. "Sham" controls were performed by adding the bathing solution that did not contain PTH or any other agents.

4. Materials

PTH fragments, including bPTH-(1-34), bPTH-(3-34) and bPTH-(7-34), were purchased from Bachem Inc. (Torrance, CA, USA). They were dissolved in distilled water and 15 μl fractions of the stock solution of 2.43 x 10^{-4}M were stored at -80°C. Oxidation of bPTH-(1-34) was carried out with H_2O_2 (Pang *et al.*, 1983).

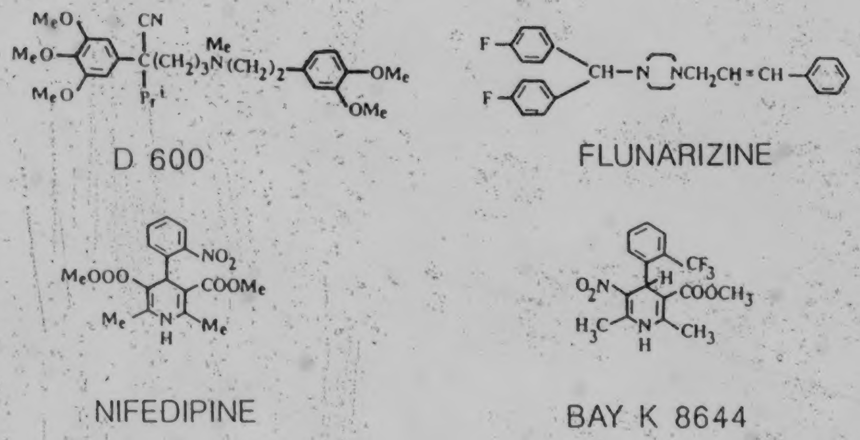
Bay K-8644 (Calbiochem) was dissolved in absolute ethanol to make a 2 x 10^{-3}M stock solution. Nifedipine (Sigma) was dissolved in acetone and $LaCl_3$ (Sigma) was dissolved in distilled water. D-600 (Calbiochem) and flunarizine (Sigma) were dissolved in DMSO and diluted further with ethanol.

Dibutyryl adenosine 3':5'-cyclic monophosphate (db-cAMP) and imidazole were purchased from Sigma. Rp-cAMPs was from Biolog Life Science Institute (FRG).

The required concentration was obtained in a 3 ml bath dish by adding 5 to 15 μl of stock solution to the dish. In the control experiments, the same volume of either bath solution, ethanol or acetone was added to the bath dish. The final concentration of solvents, ethanol (0.27%) and acetone (0.3%), when added to the bath solution had no detectable effect on the magnitude or kinetics of the inward current. The final concentration of bPTHs in the bath was achieved by a single addition in order to avoid possible desensitization of the cell to the peptide. Only one experiment was conducted per dish. The wash-out protocol was executed to examine the reversibility of the effects of the agents on calcium channel currents. Satisfactory washout of the agents from the bath solution was obtained with a perfusion rate of approximately 1 ml/10 sec.

5. Statistics

Unless original traces or single experiments are shown, values are expressed as means ± SE. The data from studies on the peak inward current amplitude are expressed as either absolute values or percentage of the pre-drug control condition. In most cases, it is the peak inward current that is used in the figures. The paired Student's t-test or group t-test was used for comparison between mean values of the control and those obtained after drug administration. In the case of multiple comparisons, analysis of variance in conjunction with the Newman-Keul's multiple range test was applied. Values of $p < 0.05$ were considered statistically significant.


MeO
MeO
MeO
CN
Me
C(CH2)3N(CH2)2
Pr i
OMe
OMe
F
F
CH—N NCH2CH=CH
NO2
MeOOO
COOMe
Me
N
H
Me
CF3
O2N
H
COOCH3
H3C
N
H
CH3

D 600

FLUNARIZINE

NIFEDIPINE

BAY K 8644

Fig. II-1. Structures of some calcium channel antagonists and an agonist.

CHAPTER 3

Characteristics of voltage-dependent calcium channels in neuroblastoma cells

INTRODUCTION

The neuroblastoma (N1E-115) cell is a useful model for studying the characteristics of calcium channels and for examining the effects of drugs and hormones (Freedman *et al.*, 1984a; Quandt and Narahashi, 1984). Although many studies utilizing voltage clamp techniques had demonstrated a single population of calcium channel currents in N1E-115 cells (Moolenaar and Spector, 1978; Bolsover, 1986), two types of voltage-dependent calcium channel currents were not demonstrated until 1987 (Narahashi *et al.*, 1987). The type I channel is characterized by an inward current which, upon depolarization, activates and inactivates quickly. The type II channel is characterized by an inward current which is activated at more positive potentials than the type I channel and shows little or no inactivation. The type II channel activity is increased by an elevation of the intracellular level of cAMP, while the type I channel is not significantly affected.

In the present study, two types of calcium channels in N1E-115 neuroblastoma cells are described as T and L channels. The T channel is similar to the type I and the L channel to the type II, as defined by Narahashi *et al.* (1987). Following the nomenclature of several previous publications from this laboratory (Pang *et al.*, 1990; Wang *et al.*, 1990a), the terms T and L channels are used in this thesis for consistency. Since the activation ranges for T and L channels in neuroblastoma cells overlap, it is difficult to study the characteristics of these two channels independently. Further manipulation is required in order to isolate cells with predominantly T or L channel currents.

RESULTS

N1E-115 cells cultured in media with or without 2% DMSO had a different morphological appearance (Fig. III-1). The cells cultured in DMEM without DMSO had a smooth surface and were spheroidal in shape (Fig. III-1A). Cells cultured in DMEM with 2% DMSO differentiated more rapidly. After culture in DMEM with 2% DMSO for approximately one month, N1E-115 cells started to sprout. Simultaneously, the viability of cells decreased and the cell membrane became more leaky. Hence, it is not easy to obtain cells which expressed predominantly L channels. A typical cell with predominantly L channels is shown in Fig. III-1B. The soma of this cell has three visible neural outgrowths (neurites). Detailed culture methods are described in Chapter 2.

Transient inward Ba^{++} currents (T channel currents).

Fig. III-2 shows the current records (leakage not corrected) and the associated current-voltage (I-V) relationship in one cell cultured in medium without 2% DMSO. Step depolarizations from a holding potential of -80 mV to potentials more positive than -50 mV activated an inward current which peaked at test potentials in the range of -20 to -10 mV. This inward current activated (time to peak, 8 to 20 msec) and inactivated quickly (half time, 40 to 70 msec), and completely inactivated at the end of a 200 msec pulse. The reversal potentials for this current ranged between +40 to +60 mV.

Long-lasting inward Ba^{++} currents (L channel currents).

In cells cultured for more than one month in medium containing 2% DMSO, depolarizations to potentials more positive than -20 mV elicited a long-lasting inward Ba^{++} current (holding potential -40 mV). During the 200 msec test pulse this current did not inactivate appreciably. Increasing the test pulse to 400 msec did not reveal any additional inactivation. The peak inward current occurred at +10 to +20 mV. Fig. III-3 shows the original L channel current records (leakage corrected) and the associated I-V relationship in one cell. These findings are almost the same as reported by Narahashi *et al.* (1987). The reversal potentials for L channel currents also ranged between +40 and +60 mV.

Some of the cells which were cultured in media containing DMSO for less than 3 weeks occasionally expressed both types of inward currents. This is also consistent with the results reported by Narahashi *et al.* (1987). Fig. III-4 shows the I-V relationship of T and L channels which were expressed in the same cell. The T and L channels of these cells had the same I-V relationships as those in cells with predominantly T or L channels.

The selective amplification of L channel currents by Bay K-8644.

Bay K-8644 can enhance calcium or barium currents by means of increasing the frequency of opening and prolonging the mean open time of the single Ca^{++} channel (Fox *et al.*, 1984a; Kokubun and Reuter, 1984). Bay K-8644 acts specifically on L channels and has no effect on T channels, as reported for neuronal cells by Nowycky *et al.* (1985) and cardiac cells by Hess *et al.* (1984). Detailed observations regarding the actions of Bay K-8644 on different calcium channels in N1E-115 cells have not been reported.

The experiment illustrated in Fig. III-5 shows that Bay K-8644 at a concentration of 1 μM almost doubled the L channel currents. Bay K-8644 at a concentration of 5 μM increased the amplitude of the L channel currents by a factor of nearly two (184% as compared to the control value of 100%, $p< 0.05$) (Fig. III-6). Tail currents through the L channel (Fig. III-5) were also enhanced. In N1E-115 cells, Bay K-8644 did not shift the I-V relationship as has been reported by Hess *et al.* (1984), and no change in activation and inactivation kinetics were observed (Fig. III-5). In most of the cells studied, an increase in leakage current occurred in the presence of Bay K-8644. As shown in Fig. III-5, Bay K-8644 had no effect on T channel currents in N1E-115 cells. Similar results were reported in neuronal cells (Nowycky *et al.*, 1985) and in cardiac cells (Bean, 1985).

The selective inhibition of L channel currents by nifedipine.

Nifedipine selectively blocked L channel currents in neuroblastoma cells while T channel currents were unaffected. These results are in agreement with those reported by Loirand *et al.* (1986) and Bean *et al.* (1986). Fig. III-7 shows the effect of nifedipine on T and L channel currents at a concentration of 10^{-4}M. In six cells, the amplitude of L channel currents was reduced to 65 ±6% ($p<0.05$). In another four cells, the amplitude of L channel currents was reduced to 16 ±4% ($p<0.05$) in response to 3×10^{-4}M nifedipine. The time course and I-V relationships of L channel currents were not changed by nifedipine. Comparison of peak inward T channel currents before and 5 to 10 min after the application of nifedipine showed no significant differences ($p>0.05$) (Table III-1).

The effect of D-600 on T and L channel currents

It has been reported that D-600 only blocked the L channel but had no effect on the T channel. In the present study, it was found that D-600 blocked both the T and L channels to almost the same degree (Fig. III-8). In the case of T channel currents, D-600 at the concentrations of 5×10^{-5}M and 10^{-4}M decreased the amplitudes of peak currents to 66% and 13%, respectively. Fig. III-9 shows that the inward currents were decreased as the concentration of D-600 was increased and were completely blocked in the presence of 2 mM La^{+++}. The basic shapes of the I-V plots of T channel currents were unchanged in the presence of D-600.

The effects of calcium channel antagonists and agonists on the T and L channel currents are summarized in Table III-1.

DISCUSSION

Calcium channel currents in neuroblastoma cells.

In order to study the currents through Ca^{++} channels in N1E-115 cells quantitatively, it is important to eliminate currents through K^{+} and Na^{+} channels. Several manipulations were used to ensure that the inward currents measured were via calcium channels. The composition of the bath solution and pipette solution was chosen to eliminate any possible contamination of sodium and potassium channel currents (see Chapter 2). 2 mM La^{+++}, an inorganic blocker of calcium channels, or 4 mM EGTA, an chelator of divalent cations (Ca^{++} and Ba^{++}), completely eliminated the inward currents, which demonstrated that the inward currents were either via the calcium channels or were carried by Ca^{++}/Ba^{++}. The organic Ca^{++} channel antagonists nifedipine and D-600 blocked the inward currents. These results helped to identify the Ca^{++} channels. In brief, the inward currents in the present studies were verified to be the barium inward current via calcium channels.

The manipulation of different culture conditions described in this chapter easily separated the N1E-115 cells with predominantly T channel currents from those with predominantly L channel currents. This method facilitated the study of L or T channel currents individually. The success of the separation of T and L channel currents by the culture method is substantiated by patch clamp studies.

1) Electrophysiological evidence: The threshold potential for the activation of the T channel was about -50 mV, and the maximum amplitude of T channel currents was obtained at potentials positive to -10 mV with an extrapolated reversal potential from +40 to +60 mV. Inactivation was rapid. The properties of this T channel are comparable to those variously described as type I (Narahashi *et al.*, 1987), transient-type (Nowycky *et al.*, 1985), SD channel (slowly deactivating) (Armstrong and Matteson, 1985), LVA channel (low-voltage activated) (Carbone and Lux, 1984), Ca I channel (Fox and Krasne, 1984b) or fast channel (Fedulova *et al.*, 1985).

The L channel has been well characterized in different tissues, such as in pituitary cells (Matteson and Armstrong, 1986; Cohen and McCarthy, 1987), hippocampal neuronal cells (Yaari *et al.*, 1986), *Neanthes* egg cells (Fox and Krasne, 1984), rat d.r.g. neurons (Fedulova *et al.*, 1985), vascular smooth muscle cells (Sturek and Hermsmeyer, 1986), skeletal muscle cells (Cognad *et al.*, 1986), cardiac muscle cells (Nilius *et al.*, 1985) and neuroblastoma cells (Tsunoo *et al.*, 1984; Narahashi *et al.*, 1987). Consistent with other reports, the L channel observed in this chapter was electrophysiologically different from the T channel. It was activated by stronger depolarizations, i.e. more than -20 mV, and the peak current amplitude occurred at membrane potentials from +10 to +20 mV. This current did not inactivate appreciably.

2) Pharmacological evidence: In agreement with the report by Freedman and Miller (1984b), Bay K-8644 enhanced L channel currents in N1E-115 cells. However, the presence of this calcium agonist did not shift the peak of the current-voltage plots. This is similar to the results of Yatani *et al.* (1987b) using the isolated smooth muscle cells of the dog saphenous vein. Nifedipine, however, blocked L channel currents in N1E-115 cells without affecting the kinetics. This has also been reported for smooth muscle cells (Bean *et al.*, 1986). As expected, neither Bay K-8644 nor nifedipine affected the T channel currents. La^{+++} and D-600 block both of these two channels. A detailed analysis of this aspect is presented later.

The above electrophysiological and pharmacological characterization strongly suggest the existence of two independent populations of calcium channels rather than a single channel type with two modes of behaviour.

N channel currents have been reported in sympathetic neurons (see review by Miller *et al.*,1988). However, in N1E-115 cells, a tumor line of sympathetic ganglia neuron, N channel currents have not been identified (Ogata and Narahashi, 1990; Audesirk *et al.*, 1990). The difference in expression of different types of calcium channel between a normal sympathetic neuron and a tumor line of sympathetic neuron is obvious. T channel currents can be identified in N1E-115 cells but not in normal sympathetic neurons (see review by Miller *et al.*, 1988). Hence, the absence of N channels in N1E-115 cells can be envisioned. The dihydropyridine sensitivity of L channel currents in N1E-115 cells also helps to separate L from N channel currents. However, the existence of N channels in N1E-115 cells during various stages of differentiation cannot be completely excluded. Some of the reasons are the difficulty of separation of L from N channel currents based on the kinetics of inactivation and the failure to use ω-conotoxin as a selective blocker for N channel currents (see review by Hess, 1990).

Selective modifications of L channel currents by dihydropyridines.

It has been reported that the dihydropyridine compound Bay K-8644, a nifedipine analogue (Fig. II-1), enhanced Ca^{++} entry or Ca^{++}-dependent events (Freedman and Miller, 1984b) in neuroblastoma cells. In cell-attached patch recording of single Ca^{++} channel activity, Bay K-8644 strongly increased the frequency of long opening of single channels in neurones (Fox *et al.*, 1984). This chapter presents the first report of the selective modification of L channel currents in N1E-115 cells by Bay K-8644 using the whole-cell version of the patch clamp technique. The effect of Bay K-8644 on N1E-115 cells may be due to either an increase in the probability of the channel being open by prolonging the mean open time (Hess *et al.*, 1984; Martha *et al.*, 1985) or an increase in the probability of re-opening of closed channels (Brown *et al.*, 1984) rather than an increase in the amplitude of the single channel current. The increased amplitude of the tail currents (Fig. III-5) in the presence of Bay K-8644 may also be due to an enhancement by the agonist of the probability that the Ca^{++} channel will be open (Droogmans and Callewaert, 1986).

The most significant structural difference between Bay K-8644 and nifedipine is that the ester group of nifedipine at position 3 of the dihydropyridine ring is substituted by a NO_2 group to form the Bay K-8644 molecule (Fig. II-1). Godfraind (1982) indicated that the nifedipine-evoked inhibitions of rat aorta and mesenteric artery contraction and $^{45}Ca^{++}$ uptake can be related to blockade of calcium entry through channels opened during depolarization or receptor-response coupling. Further studies using the patch clamp technique show that, in smooth muscle cells of rat portal vein and mesenteric artery, L channels could be blocked by nifedipine but T channels were insensitive to this drug (Bean *et al.*, 1986; Loirand *et al.*, 1986). So far no electrophysiological study on the effect of nifedipine on the two types of calcium channels in N1E-115 cells is available. The results shown in this chapter demonstrated that nifedipine selectively blocked the L channel current whereas the kinetics of this channel current were not changed. The T channel was unaffected by nifedipine or Bay K-8644.

The L channel possesses three drug binding sites which are specific for dihydropyridines, phenylalkylamines and benzothiazepines (Ruth *et al.*, 1985; Triggle and Janis, 1987). It is not surprising that D-600, a phenylalkylamine, blocked L channels. The comparison between nifedipine and D-600 shows that, although both block the L channel, only D-600 is effective in blocking the T channel. At concentrations of 50 and 100 μM, D-600 reduced the T channel currents to 66% and 13%, respectively. In contrast, it has been reported (Moolenaar and Spector, 1979) that T channel currents and Ca^{++} spikes or the prolonged after-hyperpolarization in N1E-115 cells cannot be blocked by 20 μg/ml verapamil or D 600 (approximately 35 μM). The discrepancy between their results and ours may be due to different culture conditions. In their experiments, the cells were maintained in confluence for 3 to 4 weeks, treated with trypsin and grown in 2% DMSO-containing medium. These conditions were different from the conditions used in the present study when the T channel was under investigation. In addition, the concentrations of D-600 used were different.

There are several explanations for the observation that high concentrations of calcium antagonists were needed to affect L or T channel currents in N1E-115 cells (i.e. 10 to 100 μM). First, the distribution of the subtypes of voltage-dependent calcium channels shows a larger degree of tissue selectivity. The sensitivities of these channels to antagonists in different tissue preparations may also be different. The calcium channels in neuronal tissue have been reported to be quite insensitive to organic calcium channel blockers (Louvel *et al.*, 1986). Gurney and Nerbonne (1984) reported that 100 μM nifedipine had no effect on calcium channel currents in cultured chick ciliary neurons or in rat superior cervical ganglion cells where the existence of L channels was well documented (see review by Miller *et al.*, 1988). Usually, D-600 and verapamil block Ca^{++} currents or Ca^{++}

spikes only at high concentrations (> 100 μM) in nervous tissue. Douglas and Taraskevich (1982) used 10 μM D-600 in rat pars intermedia cells but this concentration could not inhibit Ca^{++} spikes. In view of these reports, the concentration range from 10 to 100 μM used to block T or L channels in N1E-115 cells is acceptable. In addition, different solvents can alter the effectiveness of dihydropyridines on L channel currents. In the present study, acetone was used to dissolve nifedipine. Using DMSO as the solvent, nifedipine can block L channels in N1E-115 cells by 50% at a concentration of 0.1 μM (Wu LY, Wang R, Karpinski E, Pang PKT, unpublished observation, 1990). Second, there is controversy regarding the site(s) of action of calcium channel antagonists. For example, Heschler *et al.* (1982) pointed out that D-600 inhibited the Ca^{++} channel from the internal surface of cardiac muscle cell, whereas Ohya *et al.* (1987b) demonstrated that D-600 blocked Ca^{++} channels in smooth muscle cells from the outer surface. If the site(s) of action of D-600 and nifedipine were located on the inside surface of the N1E-115 cell membrane, there is no doubt that a higher concentration would be needed for drug permeation to the inner surface of the cell. Third, in the present study, the selective inhibition of L channel currents by nifedipine can clearly be demonstrated using this concentration range (10 to 100 μM). This selective inhibition eliminated the possibility of a nonspecific action of higher concentrations of the antagonist on the calcium channel currents.

A

B

Fig. III-1. Morphological appearance of N1E-115 cells cultured in different culture media. **A.** Cells were cultured in DMEM without DMSO. These cell expressed only T channel currents. A cell and pipette (whole cell configuration) is shown in the right top corner. **B.** One cell was cultured in DMEM with 2% DMSO. This cell expressed only L channel currents. Phase contrast photomicrographs were taken the day of channel recording. Bar represents 50 μm.

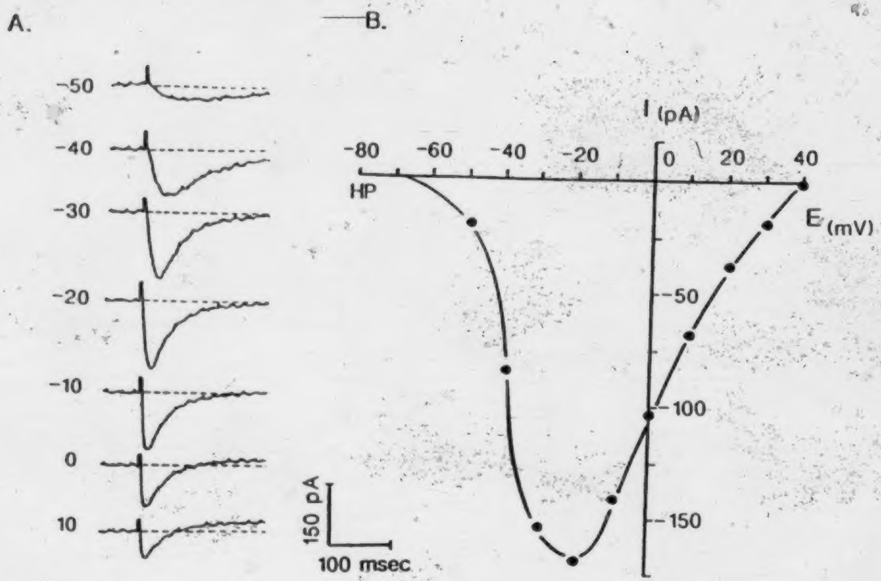

A.
B.
-50
-40
-30
-20
-10
0
10
150 pA
100 msec
I (pA)
-80
-60
-40
-20
0
20
40
HP
E (mV)
-50
-100
-150

Fig. III-2. Current records (**A**) and the associated current-voltage relationship (**B**) in an N1E-115 neuroblastoma cell which expressed predominantly T channels. The holding potential was -80 mV and the magnitude of the test potentials are indicated beside each current record (**A**). Zero current levels are indicated by the dashed lines. The original current records are not leakage or transient corrected. The current-voltage relationship is leakage corrected.

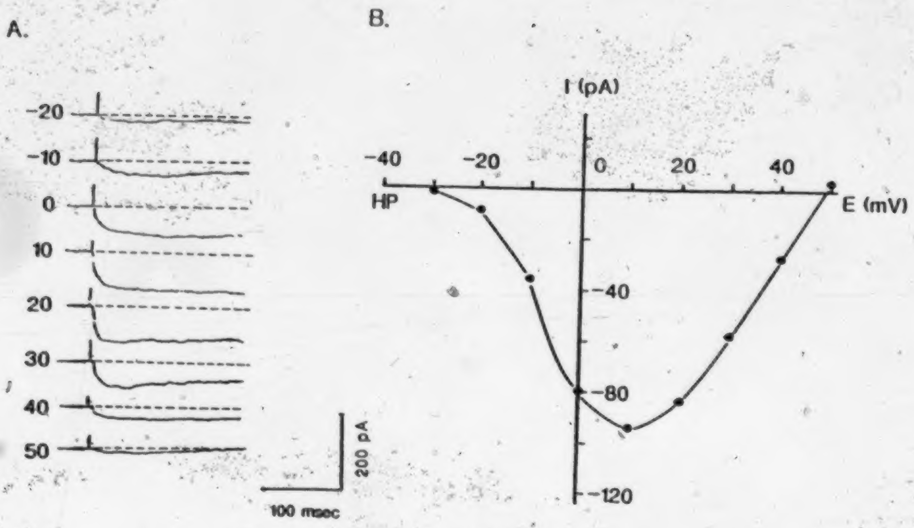

A.
-20
-10
0
10
20
30
40
50
200 pA
100 msec
B.
I (pA)
-40
-20
0
20
40
HP
E (mV)
-40
-80
-120

Fig. III-3. Current records (**A**) and the associated current-voltage relationship (**B**) in an N1E-115 neuroblastoma cell which expressed predominantly L channels. The holding potential was -40 mV and the magnitudes of test potentials are indicated beside each current record (**A**). Zero current levels are indicated by the dashed lines. The original current records are leakage and transient corrected. This cell was cultured for 4 weeks in a medium with 2% DMSO.

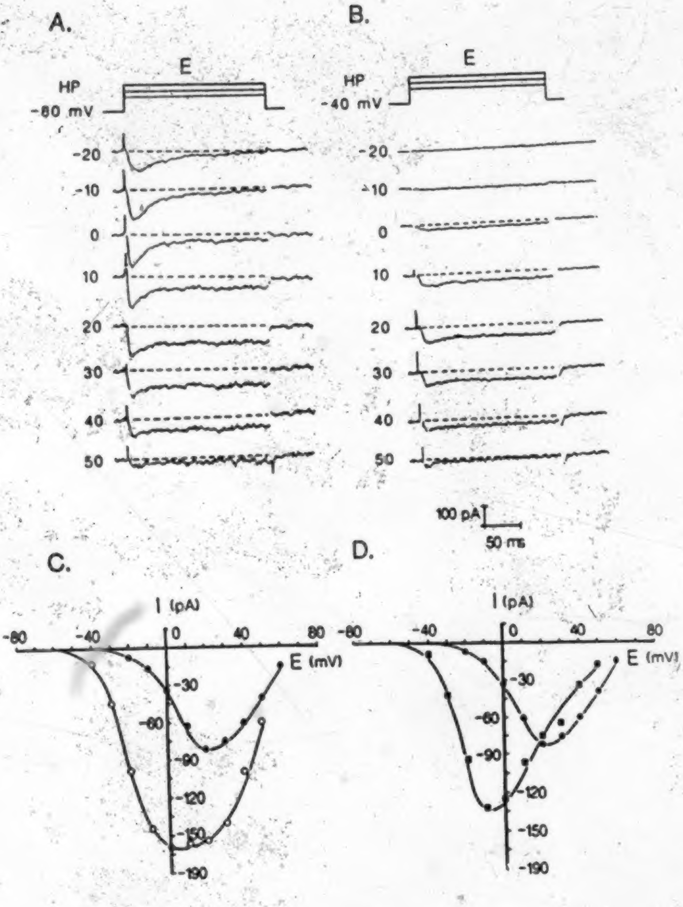

A.
B.
E
HP
-80 mV
-40 mV
-20
-10
0
10
20
30
40
50
100 pA
50 ms
C.
D.
I (pA)
E (mV)
-80
-40
0
40
80
-30
-60
-90
-120
-150
-190

Fig. III-4. Current records (**A** and **B**) and the associated current-voltage relationships (**C**, **D**) in an N1E-115 cell which expressed both T and L channels. **A.** The holding potential was set at -80 mV. The original current records (leakage and transient corrected) are shown with the magnitude of the test potential indicated beside each current record. Zero current levels are indicated by the dashed lines. These current records contain both T and L channel components. The I-V relationship is shown in **C** (open circles). **B.** The holding potential was shifted to -40 mV in the same cell as in **A.** The current elicited was a characteristic L channel current. The corresponding I-V relationship is shown in **C** (filled circles). Subtraction of the L channel I-V relationship from the total I-V relationship results in the I-V relationship of the T channel current, shown in **D** (filled squares). Shown for comparison in **D** as filled circles is the L channel I-V relationship. This cell was cultured in a medium with 2% DMSO for 3 weeks.

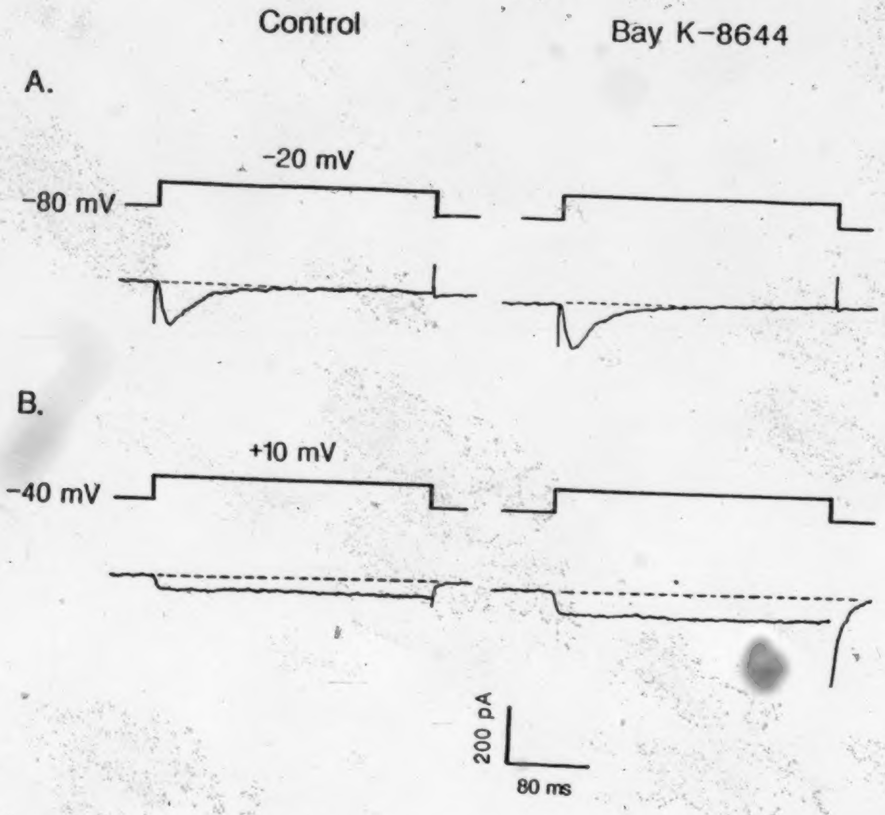

Control
Bay K-8644
A.
-20 mV
-80 mV
B.
+10 mV
-40 mV
200 pA
80 ms

Fig. III-5. Different effects of Bay K-8644 (1 μM) on T and L channel currents in N1E-115 cells. **A.** Records before and 5 min after application of Bay K-8644. The holding potential was -80 mV. **B.** Records before and 5 min after Bay K-8644. The holding potential was -40 mV. The L channel current and tail current were increased by Bay K-8644. Leakage currents have been subtracted from the original current records.

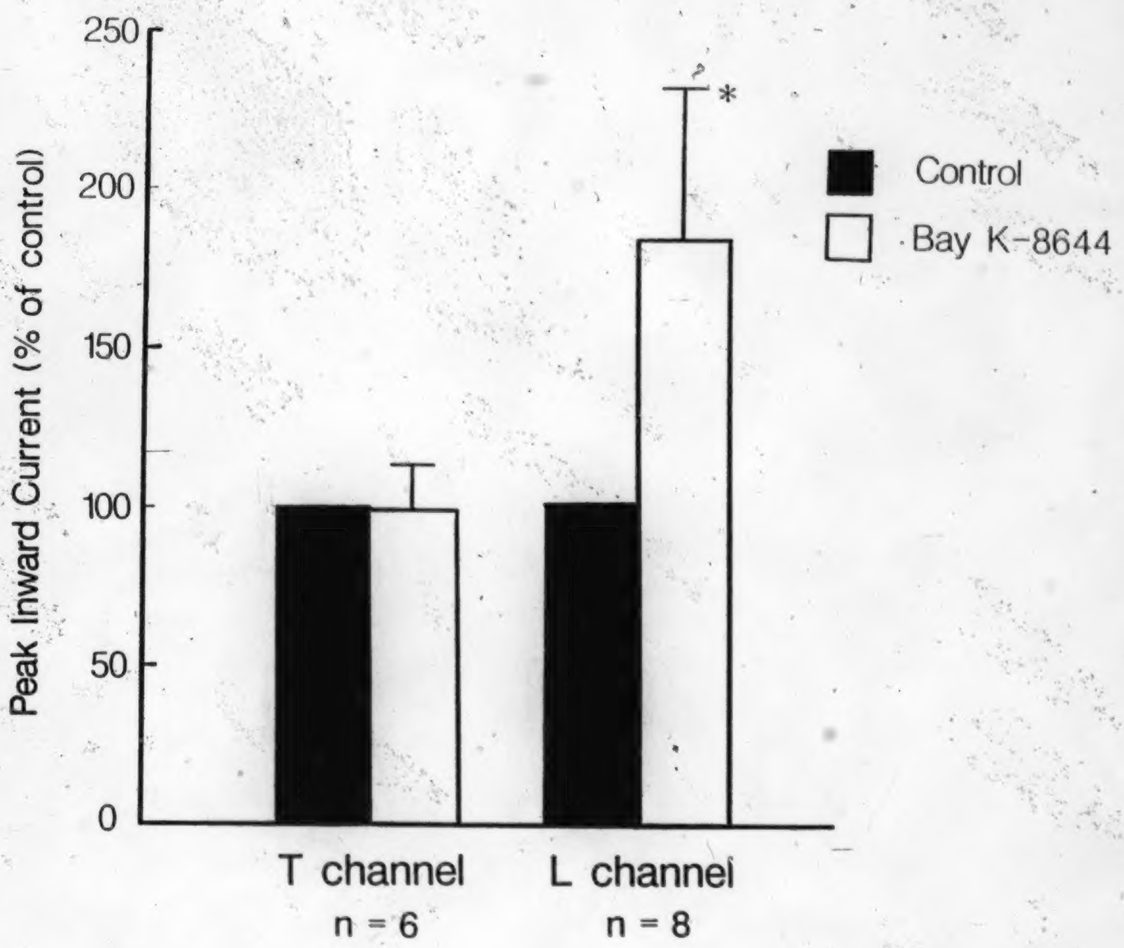

Peak Inward Current (% of control)
250
200
150
100
50
0
*
Control
Bay K-8644
T channel
n = 6
L channel
n = 8

Fig. III-6. The sensitivities of T and L channels in N1E-115 cells to Bay K-8644. The peak inward currents in the presence of 5 μM Bay K-8644 were measured 5 to 7 min after the drug was given and are expressed as the I_{peak}. The corresponding value of the control ($I_{control\ peak}$) was 1.00. In the experiments with T channels, n=6; for L channels, n=8. Values are means ± SE. * indicates $p < 0.05$.

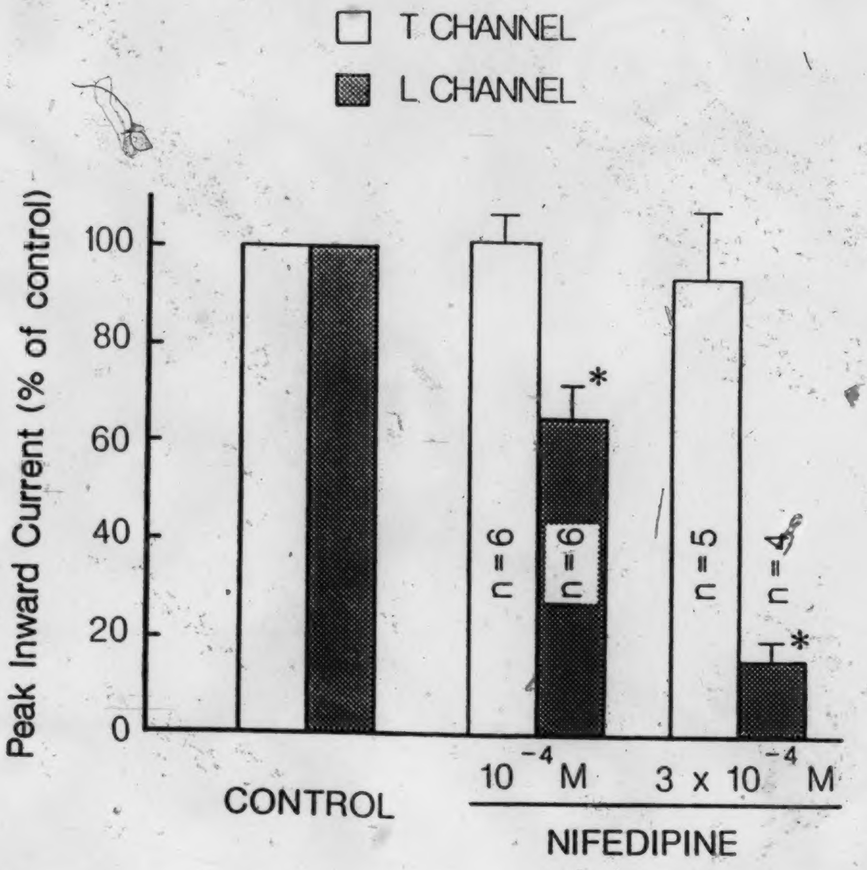

☐ T CHANNEL
▨ L CHANNEL
Peak Inward Current (% of control)
100
80
60
40
20
0
n = 6
n = 6
n = 5
n = 4
*
*
CONTROL
10⁻⁴ M
3 x 10⁻⁴ M
NIFEDIPINE

Fig. III-7. The effects of nifedipine on the peak inward currents of T and L channels in N1E-115 cells. Nifedipine at two different concentrations inhibited L channel currents, but not T channel currents. The amplitude of the inward currents in the absence of nifedipine was taken as 100%. * indicates $p<0.05$.

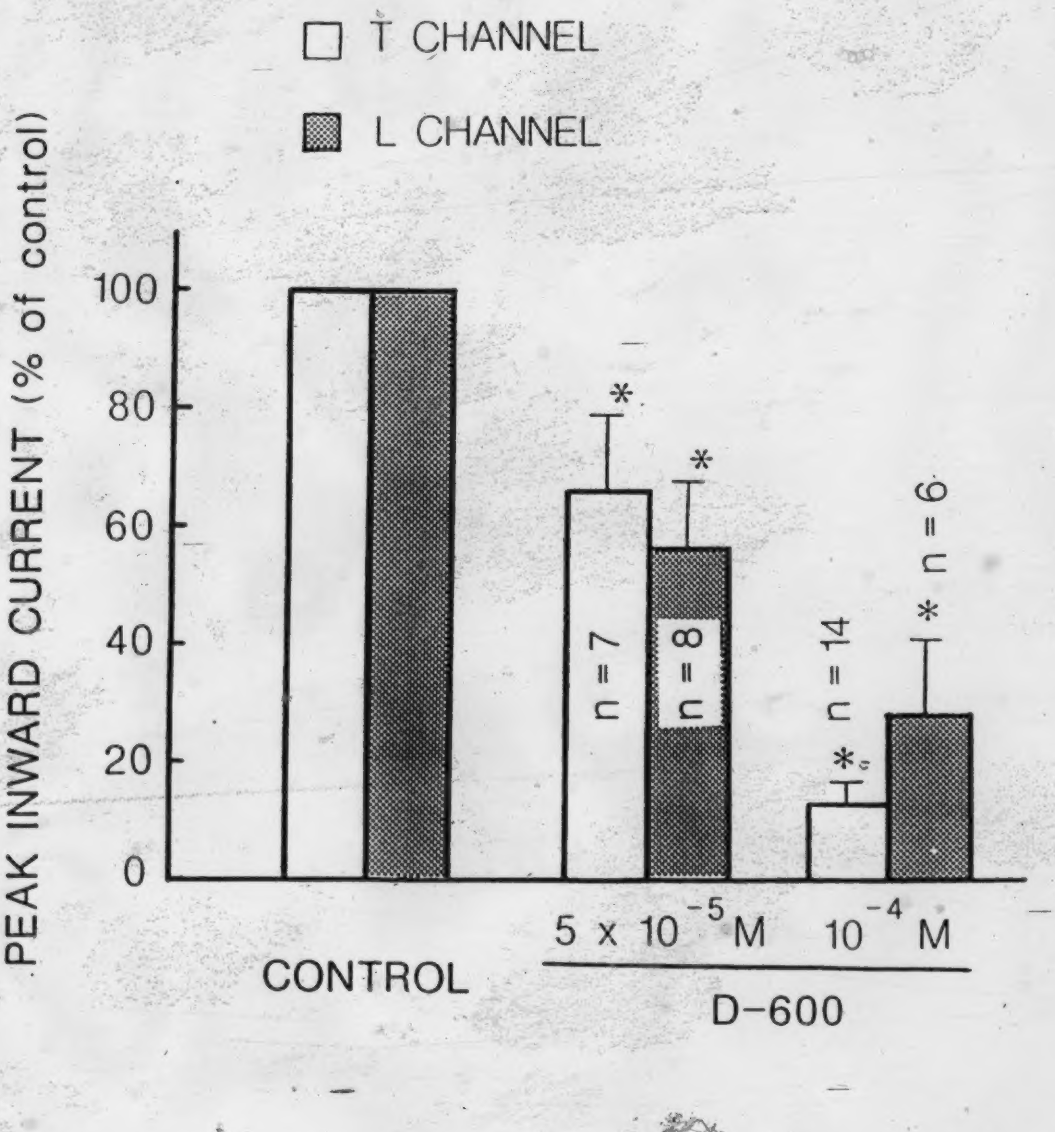

T CHANNEL
L CHANNEL
PEAK INWARD CURRENT (% of control)
100
80
60
40
20
0
n = 7
n = 8
n = 14
n = 6
*
CONTROL
5 x 10⁻⁵ M
10⁻⁴ M
D-600

Fig. III-8. The effects of D-600 on the peak inward currents of T and L channels in N1E-115 cells. D-600 at two different concentrations inhibited both L and T channel currents. The amplitude of the inward currents in the absence of D-600 was taken as 100%. * indicates $p<0.05$.

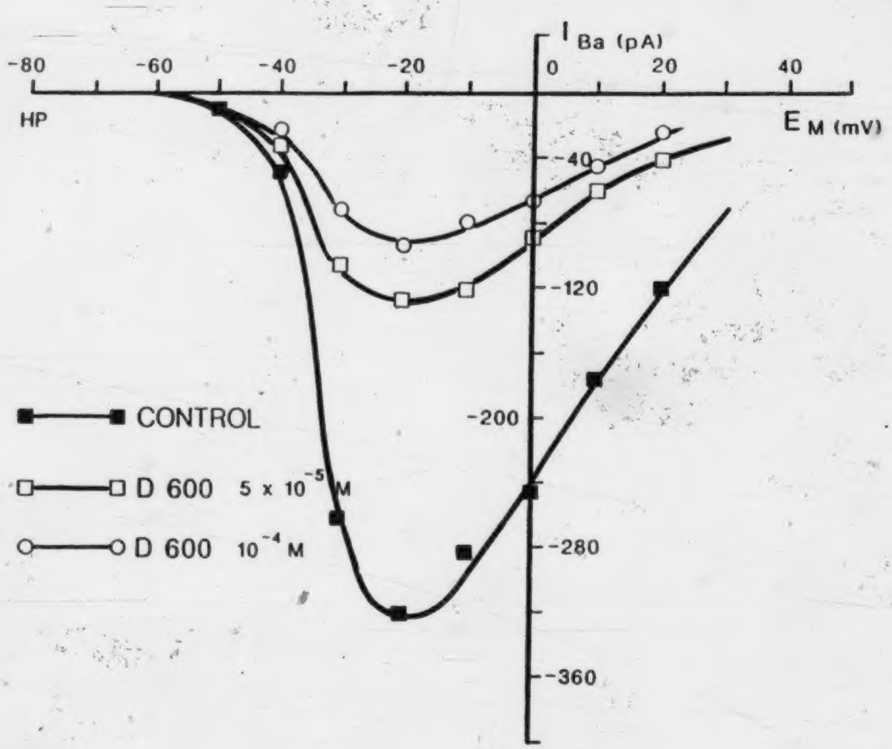

I Ba (pA)
-80
-60
-40
-20
0
20
40
HP
E M (mV)
-40
-120
-200
-280
-360
■—■ CONTROL
□—□ D 600 5 x 10^-5 M
○—○ D 600 10^-4 M

Fig. III-9. The effect of D-600 on T channel currents in one N1E-115 cell. I-V relationships of T channel currents is constructed from the same cell. The leakage currents have been subtracted. D-600 decreased the amplitude of the T channel current but did not change the I-V relationship.

Table III-1:

Effects of calcium channel antagonists and an agonist on peak inward currents (% of control) in N1E-115 cells

Agents	Concentration	T Currents	P values*	L Currents	P values*
Control		100		100	
Bay K-8644	5 μM	95 ± 13	>0.05	184 ± 47	<0.05
D-600	50 μM	66 ± 13	<0.05	56 ± 10	<0.05
	100 μM	13 ± 4	<0.05	28 ± 13	<0.05
Nifedipine	100 μM	101 ± 6	>0.05	65 ± 7	<0.05
	300 μM	94 ± 14	>0.05	16 ± 4	<0.05
$LaCl_3$	2 mM	0	<0.05	0	<0.05

* When compared with the control values.

CHAPTER 4

Characteristics of voltage-dependent calcium channels in rat tail artery smooth muscle cells

INTRODUCTION

Since the development of the patch clamp technique and the first success in the isolation of smooth muscle cells (Bagby *et al.*, 1971), knowledge of the distribution, structure, and function of voltage-dependent calcium channels in single vascular smooth muscle cells has advanced. Thus far, two types of time- and voltage-dependent calcium channels have been identified in arterial smooth muscle cells, including those from rabbit ear artery (Benham *et al.*, 1987), rat mesenteric artery (Bean *et al.*, 1986), and guinea pig aorta (Cafferey *et al.*, 1986). The transient (T) current, which activates quickly and then inactivates, may contribute to the generation of smooth muscle cell action potentials. The long lasting (L) current, which displays little or no inactivation, is thought to be important for excitation-contraction coupling. The distribution of these calcium channels in various arterial vascular beds may be related to the different functional roles of these arteries.

Studies by Pang *et al.* (1984) in which strips of the rat tail artery were used have suggested indirectly the existence of voltage-dependent calcium channels in these smooth muscle cells. They reported that D-600 and bPTH-(1-34) inhibited the tension elicited by KCl in rat tail artery strips. The action of KCl has been ascribed to the depolarization of the cell membrane, which in turn activates surface membrane calcium channels (Hurwitz *et al.*,1980; Brading et al.,1983; Huddart and Butler, 1983; Karaki, 1987; Langton and Huddart, 1987). The contraction of smooth muscle from the rat tail artery in the presence of a high concentration of KCl, hence, is initiated by the opening of voltage-dependent calcium channels. In addition, calcium-induced calcium release from sarcoplasmic reticulum may also participate in the maintenance of contractile activity due to the KCl challenge (see review by van Breemen and Saida, 1989).

Using the Ca^{45} uptake technique, Pang *et al.* (1984) found that KCl stimulated calcium uptake by rat tail artery *in vitro* and that D-600 decreased this effect. However, there is no electrophysiological evidence demonstrating the existence of calcium channels in smooth muscle cells from the rat rail artery. The present study was undertaken to determine whether calcium channels exist in single smooth muscle cells from rat tail artery and, if they do exist, to investigate the electrical properties and pharmacological characteristics of these channels. By combining the whole cell configuration of the patch-clamp technique with short-term primary culture, a fast-inactivating calcium channel current and a separate slow-inactivating and dihydropyridine-sensitive calcium channel current have been identified. Two types of calcium channels in rat tail artery smooth muscle cells were also reported by Bolzon *et al.* (1988).

The characteristics of the two types of calcium channel currents in rat tail artery smooth muscle cells are consistent with other reports using smooth muscle cells from rabbit ear artery (Benham *et al.*, 1987; Aaronson *et al.*, 1988) and saphenous vein (Yatani *et al.*, 1987b). In all of the above reports the experiments were carried out at room temperature (20°-25°C). Since the characteristics of calcium channels at *in vivo* temperature may not be the same as those at room temperature, it is important to examine calcium channel currents in vascular smooth muscle cells at different temperatures and to compare the temperature dependence of calcium channel currents reported for other preparations. The temperature dependence of L channel currents in ventricular myocytes (Cavalie *et al.*, 1985) and neurons (N1E-115 cells) (Narahashi *et al.*, 1987) have been well documented. Therefore, the study of the temperature dependence of L channel currents in my thesis was focused on vascular smooth muscle cells because of the lack of knowledge concerning this type of cell preparation.

It has been reported that some dihydropyridines affected L channels in a temperature-dependent manner (Hosey and Lazdunski, 1988). In addition, the binding of antagonists to calcium channel receptors in vascular smooth muscle has been shown to be temperature-dependent (Papaioannou *et al.*, 1986). Hence, an understanding of the temperature dependence of L channels in smooth muscle cells will also help elucidate the mechanism(s) of pharmacological regulation and may facilitate the biochemical purification of L channel proteins. Hence, in this chapter, the temperature dependence of the amplitude, the half-times of activation and inactivation, the current-voltage relationship and the "run-down" of the L channel currents are also described.

RESULTS

Morphology of freshly dispersed smooth muscle cells

Immediately after harvest, the smooth muscle cells were spheroidal or oval in shape (Bean *et al.*, 1986), 14-32 μm long and 14-24 μm wide in the central portion. Dispersed cells attached to the dish during the first 6 hr of culture. After 2-3 days they spread out and became elongated and spindle shaped.

In our preparation, almost all the harvested cells were smooth muscle cells.

The evidence to support this statement is as follows. 1) The cell cytoplasm was phase dense and homogeneous and had smooth and bright borders as observed with phase contrast microscopy (Chamley-Campbell *et al.*, 1979). 2) The cells did not contract spontaneously but had a good contractile response when touched by electrodes or exposed to 10 µM norepinephrine (Ives *et al.*, 1978; Nakazawa *et al.*, 1987). Usually, the stimulated cells were fully and evenly contracted with bulbous projections (Fay and Delise, 1973) and at times they pulled away from the dish bottom. 3) All these oval or round cells were stained red in a manner similar to their parent tissue by Masson's trichrome stain (Marvin *et al.*, 1979). Although fibroblasts were also present, they accounted for less than 1% of the total cell population. These cells were flat and irregular with phase-lucent cytoplasm and a large clear nucleus. They did not contract in the presence of mechanical or chemical stimulation. These cells were stained blue by Masson's trichrome method. Hence the fibroblasts were distinct from the smooth muscle cells.

In light of these morphological observations we chose only those oval or round cells for recording membrane currents in our experiments.

Calcium channel currents.

Inward currents were elicited using depolarizing pulses from two holding potentials (-80 and -40 mV). Fig. IV-1A shows a series of records of inward currents carried by barium ions in a smooth muscle cell. The holding potential was

-80 mV. The time course of this current was characterized by fast activation and fast inactivation. It reached a peak within 20 msec and decayed with a half time of about 40 msec. In 5 other cells which expressed predominantly T-channels, the half time of the decay was 46.4 ± 9.6 msec (X ± SE). This current activated at a membrane potential of -50 mV and the peak inward current occurred at -20 to -10 mV. The I-V relationship corresponding to the records in Fig. IV-1A is shown in Fig. IV-1B. In this I-V plot, the peak inward current occurs at 0 mV. This is because another type of Ca^{++} channel, the L channel, was also present in this cell and the I-V relationship is the sum of the two components. The major component of this inward current is similar to the T channel reported in other types of vascular smooth muscle (Bean *et al.*, 1986; Caffery *et al.*, 1986; Benham *et al.*, 1987). The peak inward currents were 38.4 ± 7.03 pA in 14 cells (20 mM Ba^{++} as charge carrier) in which the majority of the current was via T channels and the current reversed at +50 mV.

Fig. IV-2A shows a series of records of inward currents from a smooth muscle cell in which the holding potential was -40 mV. These records show a current which inactivates with a very slow time course with a half time of greater than 150 msec. As the test potential became more positive the time course of inactivation became faster. In 19 other cells the half time of inactivation was 156 ± 8.6 msec. This current was activated at a membrane potential of -20 mV and the peak inward current occurred at +10 to 20 mV.

To exclude the possibility that inactivation of the L-channel was due to the superimposition of a small amount of outward current carried by K^+ through K^+ channels or Ca^{++} channels (Palade and Almers, 1985) on the inward current, two manipulations were used. TEA-Cl (20 mM) was added to the bath solution to inhibit any remaining K^+ currents. In addition, the internal solution was made K-free to eliminate K^+ moving through Ca^{++} channels. This was accomplished by using succinic acid instead of the potassium salt. These manipulations did not alter the slow inactivation. It is evident, then, that the slow inactivation of inward currents is one of the intrinsic properties of this calcium channel when Ba^{++} is used as the charge carrier. Similar slowly inactivating, L-type, calcium channels have been found in other smooth muscle cells (Bean *et al.*, 1986; Benham *et al.*, 1987; Droogmans *et al.*, 1987).

The peak amplitudes of L-type calcium channel currents were 25.79 ± 5.74 pA (n = 33). A complete separation of L-type calcium channels from T-type calcium channels was difficult. The proportion of two types of calcium channels varied with dispersal procedures, culture media composition and time. Sometimes mostly T-type or L-type calcium channels were observed in a single cell while at other times both types of calcium channel currents could be seen at a holding potential of -80 mV. Fig. IV-3 shows both T- and L-type calcium channels superimposed. When the holding potential was maintained at a value more negative than -70 mV, both T- and L-type channel currents were recorded. The total current amplitudes could be measured as the peak values and the L-channel current magnitudes were defined as the value obtained at 200 msec. The T-type calcium channel was obtained from the total inward currents by subtracting the L-type channel current. When the holding potential was more positive than -60 mV, T channels were gradually inactivated and only L channels remained.

Calcium current could be demonstrated in almost all the smooth muscle cells of the rat tail artery preparations that were examined. In a total of 63 cells that were studied, 55 cells showed active calcium channel currents. The cells with predominantly L-type channels accounted for 60% and those with predominantly T-type channels represented 25% of the total cell population with calcium channels. Both T and L channels have also been observed in the same cells using two different holding potentials (-80 and -40 mV) sequentially.

Run-down of calcium channels

In the experiments described above, CsCl was used (130 mM) as the main charge carrier inside the pipette and 0.25 mM cyclic AMP was also included, since a stable calcium channel current had been successfully recorded for longer than 30 min in neuroblastoma cells using this solution. However, it was found that this was not the case for these smooth muscle cells. Both types of calcium channels were either difficult to detect or small and could be maintained for less than 5 min. In view of the rapid equilibration between the cytoplasmic and pipette solutions, Caffery and co-workers (1986) suggested that the "run-down" of calcium channel currents was due to the loss of some critical "materials" for the operation of these channels. To prolong the recording, Cs_2-aspartate was used instead of CsCl and cAMP was omitted from the pipette solution since cAMP did not prevent the "run-down" of calcium currents in the smooth muscle cells (Ohya *et al.*, 1987a). K-pyruvate and K-succinate were added to support the citric acid cycle. Phosphocreatine and creatine phosphokinase were used to facilitate the process of energy yield and utilization. As a result, in the present study, the amplitude of both T and L channel currents was increased and the inward currents could be maintained for at least 20 min. In most cells, immediately following the penetration of the cell, calcium channels were not seen. One to three minutes later, the inward currents emerged and within the following 5 min the inward currents increased and reached their peak. At the end of the 20 min recording period, the relative amplitudes of inward calcium channel currents were 0.90 ± 0.33 for the predominantly T component and 0.94 ± 0.45 for the predominantly L component, taking the peak amplitude at the start of recording as 1.00. The change in calcium channel currents with time is shown in Fig. IV-4. In several cells, the calcium channel currents were maintained for about 2 hr.

Inactivation Characteristics.

Analysis of steady-state inactivation was carried out to determine the inactivation characteristics of T- and L-type calcium channels. Those cells with predominantly T channels were depolarized to -20 mV from holding potentials ranging from -100 mV to -30 mV. In cells having predominately L channels, the holding potentials were varied from -100 mV to -10 mV with test pulses to either 0 or 20 mV. Membrane potential was first held for at least 2 sec to reach a quasi steady-state and was then followed by a test pulse of 1 sec. The peak current amplitudes during the test pulse were measured at each holding potential and normalized to the current measured at the most negative holding potential. The data were plotted as a function of holding potential and fit by the Boltzmann distribution of the form: $I = I_{max}[1 + \exp(V - V_h)/k]^{-1}$, where V is the holding potential, V_h is the test potential at which one-half of the Ca^{++} channels are inactivated,. and k is the slope.

Fig. IV-5 shows the resulting inactivation curves of T- and L-type calcium channels and representative current records. The curve (filled circles) was obtained from the T channel in one cell. The slope of the fitted curve is 6.9 mV and V_h is -50.5 mV. The T channel was inactivated at potentials positive to -30 mV. Similar steady-state inactivation curves were measured in other four cells. The inactivation curve for one cell which had predominately L channels is shown (open circles) in Fig. IV-5 (V_h = -28.6 mV, k = 8.2 mV). Similar inactivation curves were obtained in 3 other cells with predominately L channels.

Sensitivity of L-type calcium channels to dihydropyridines

The present data showed that both T- and L-type calcium channels were blocked by La^{+++} (Fig. IV-6A), while only the L channel was sensitive to the 1,4 dihydropyridine derivative, Bay K-8644 (Fig. IV-6B, upper panel). Although the composite inward currents for some cells shown in the lower panel of Fig. IV-6B also increased in response to Bay K-8644, that was only due to the increase of the L channel component. After subtraction of the non-inactivating current (i.e. the remaining current at the end of the 200 msec test pulse) the T-type channel current showed no change in the presence of Bay K-8644 (Bean *et al.*, 1986).

The effects of Bay K-8644 on the L-type calcium channel had several features. 1) Bay K-8644 increased the L-type calcium currents by more than two-fold. Fig. IV-7A shows one example in which Bay K-8644 amplified the inward currents at all test pulses and possibly increased the rate of inactivation. Fig. IV-7B shows that this agent (5 μM) displaced the peak of the I-V plot in the negative direction by 10 mV at 5 min and by 20 mV at 10 min. 2) Bay K-8644 shifted the steady-state inactivation and activation curves for L channels. Fig. IV-8 shows the changes elicited by 5 min Bay K-8644 treatment at a concentration of 5 μM in one cell. The steady-state activation curve of mostly L-type calcium channels in this cell was marked as open circles and plotted as relative peak amplitudes against test pulses. The curves were fitted with the Boltzmann equation, $I = I_{max} [1 + \exp(V_h - V)/k]^{-1}$, where V_h = -.36 mV and k = 4.8 mV. The L channel current activated at -20 mV and saturated at +20 mV in the absence of Bay K-8644 and the steady-state activation and inactivation curves crossed at -10 mV. In the presence of Bay K-8644, the steady-state inactivation curve moved 10 mV negative to that of the control and the activation curve (V_h = -10.8 mV, k = 4.8 mV) shifted in the same direction by the same magnitude as did the inactivation curve. These two curves crossed at a point between -10 and -20 mV and the overlap range was still small. In addition, Bay K-8644 did not change the slopes of either the inactivation or activation curves. 3) Bay K-8644 seemed to unmask the "quiescent" calcium channels. In 12 cells, observations lasting 10 min under whole-cell patch clamp conditions failed to show any inward current. However, following addition of Bay K-8644 (5 µM), L-type calcium channels became detectable in 10 of these 12 cells.

Furthermore, L channel currents in vascular smooth muscle cells were sensitive to dihydropyridine antagonists, such as nifedipine (Fig. IV-9).

Temperature dependence of L channel currents

The effect of temperature on the amplitudes of the peak inward L channel currents was first studied at three different temperatures (Fig. IV-10). The diameters of cells studied at 22°C, 32°C and 36°C were 18.0 ± 0.8, 17.9 ± 1.0 and 18.4 ± 1.0 µm, respectively. There was no statistically significant difference in sizes among the groups of cells studied at the various temperatures ($P > 0.05$). With the membrane potential held at -40 mV, the peak L channel current at 22°C was 70.0

± 10.8 pA (n = 17) which was significantly smaller than the peak L channel current of 212.8 ± 31.7 pA (n = 10) at 32°C ($p < 0.05$). At 36°C the peak inward current was 184.6 ± 42.0 pA (n = 8) which was also significantly larger than that at 22°C ($p < 0.05$). Since cell capacitance was not measured in this group of experiments, the changes in the current amplitude at different temperature are only qualitative and cannot be related to the current density.

Fig. IV-11A shows the original L channel inward current recorded at 22°C and 32°C in the same cell. The elevation in temperature caused an increase in both the amplitude and the time course of activation and inactivation. When the I-V relationships of L channel currents at different temperatures were compared in the same cell, no significant shift could be found (Fig. IV-11B). The activation threshold, the maximum potential at which the peak inward current occurred and the bell-shape of the I-V plots were the same at 22°C and 32°C.

To exclude the possible recruitment of channels other than the L type at higher temperatures, the effect of a specific L channel antagonist and an agonist (nifedipine and Bay K-8644, respectively) were tested at different temperatures. The inward current was almost completely blocked by 1 μM nifedipine at 32°C. A similar result with nifedipine was also obtained by Hering *et al.* (1988). When Bay K-8644 was added to obtain a final concentration of 5 μM at 32°C, the inward current was enhanced (Fig. IV-12A) (Yatani *et al.*, 1987b; Wang *et al.*, 1989). Subsequent application of 2 mM La^{+++} completely blocked the inward current (Fig.

IV-12A). These results show that an increase in temperature resulted in an increase of mainly the L-type calcium channel currents.

When the temperature was increased from 22° to 35°C, the amplitude of the inward currents increased progressively. However, when the temperature was above 35°C, the amplitude of the inward currents decreased. This is shown in Fig. IV-12B. The temperature coefficient, Q_{10}, of peak currents was 2.20 ± 0.25 ($n = 8$) and 0.56 ± 0.14 ($n = 3$) from 22° to 35°C and 36° to 40°C, respectively. Maximum current amplitude occurred at approximately 35°C.

In order to estimate the half-times of activation and inactivation, the duration of voltage-clamp depolarization was prolonged to 500 msec in another group of cells. Figs. IV-13A and 13B show the half-times ($T_{1/2}$) of inactivation and activation.

The $T_{1/2}$ of activation at 23°C was two times larger than that at 36°C (Fig. IV-13B) ($P < 0.05$). The decrease in the $T_{1/2}$ of inactivation at 36°C was also significant (Fig. IV-13A) ($P < 0.05$). The L channel current kinetics were voltage dependent. The $T_{1/2}$ of activation was 18 msec at a potential of -20 mV and decreased to 6 msec at +50 mV. At the higher temperature of 36°C the $T_{1/2}$ of activation was shorter and not as strongly voltage dependent as it was at the lower temperature (i.e. the slope was less steep). Fig. IV-13A shows the $T_{1/2}$ of inactivation plotted as a function of voltage at 23°C and 36°C. At the higher temperature the $T_{1/2}$ of inactivation decreased at all voltages and the U-shaped voltage dependence seen at 23°C became much broader, and had a shape similar to that reported by Bean (1985). The half-times of activation and inactivation were more difficult to measure at membrane potentials more positive than +50 mV because of the small inward currents generated.

At room temperature (22°C), the L channel current could be recorded for 20 min without run-down. During this time, the amplitude of the L channel currents declined at a rate of less than 2% per minute (Wang *et al.*, 1989). However, at 36°C, the L channel currents declined more than 50% within 5 min, and only 3 of 10 cells could be sustained for longer than 10 min with a 2% per minute rate of decline. As the temperature was increased, the leakage current also increased. This temperature-induced rundown of calcium channel currents in vascular smooth muscle cells was not consistent with results obtained in ventricular cells. Within 15 min, Walsh *et al.* (1989) found no difference between the rundown at room temperature and that at 32°C.

DISCUSSION

The classification of calcium channels into T and L types has been suggested to be a over simplification in vascular smooth muscle cells (Bolton et al., 1988). In vascular smooth muscle cells, T and L channel currents can be separated by their voltage-dependent activation and inactivation. However, variation of holding potentials, under some circumstances, did not give satisfactory separation of T and L channels (Aaronson *et al.*, 1988). Nevertheless, in the present study, both T and L types of calcium channel currents are satisfactorily observed and separated in 87% of the smooth muscle cells tested using the whole-cell voltage clamp technique. These two types of calcium channel currents are comparable in size, current-voltage relationships, and kinetics to those observed in earlier studies with other smooth muscle cells from the mesenteric arteries of guinea-pig (Bolton *et al.*, 1985), rabbit (Worley *et al.*, 1986) and rat (Bean *et al.*, 1986), and from rabbit ear arteries (Benham *et al.*, 1987; Droogmans *et al.*, 1987), rabbit (Ohya *et al.*, 1988) and rat portal vein (Hermsmeyer *et al.*, 1976), rat azygous vein (Sturek and Hermsmeyer, 1986) and rat tail artery (Bolzon *et al.*, 1988). However, this is the first report demonstrating the existence of voltage dependent calcium channels in smooth muscle cells of rat tail artery using the whole-cell patch clamp technique. Calcium mobilization is necessary for excitation-contraction coupling and electrical activities in smooth muscle cells of rat tail artery. Voltage-dependent calcium channels provide an important pathway for calcium entry. The successful recording of voltage-dependent calcium channels in the present study may be attributed to the following manipulations. Cells in short-term culture (8-36 hr) were used in the present study. In agreement with other reports using vascular smooth muscle cells from rabbit aorta (Ives *et al.*, 1978) and from saphenous vein (Yatani *et al.*, 1987b), the smooth muscle cells from rat tail artery were spherical or oval in shape. The rounded shape of cells may be a result of cell contraction during the dissociation process. However, these cells seemed healthy. Their surface looked smooth and did not have a "floury and fluffy" appearance. During this period of time, these cells were very sensitive to mechanical and chemical stimulation. Presumably, the cells maintained their functional state with intact calcium channels. According to my data, the calcium channels were most easily detected and were sustained for the longest time when the cells were cultured for a period of approximately 24 hr. If the culture time was longer than 72 hr, the detection of calcium channels became much more difficult. It is possible that the longer culture time may transform the functional contractile state of smooth muscle cells into the proliferative state. Consequently, the cells can no longer be induced to contract and the calcium channels can not be demonstrated (Chamley-Campbell *et al.*, 1979). The time lapse of this phenotypic modulation of smooth muscle cells differs with the tissue source.

The alteration of channel properties due to enzyme damage is another important consideration (Hermsmeyer *et al.*, 1976). In the present experiments a collagenase/dispase mixture was used. The advantage of dispase is that it maintains the integrity of cell membranes during the 1 hr incubation. In the preliminary trials, collagenase with elastase or protease alone produced a low yield of responsive cells. The addition of DNAase I, DNAase II and hyaluronidase did not significantly improve either the cell harvest or the inward current amplitude. The recording conditions also affect the detection of calcium channel currents. For example, Ohya *et al.* (1987b) reported that ATP in the pipette solution, at a concentration similar to that used in the present study, augmented the calcium channel currents by a factor of two in the smooth muscle cell myoballs of rabbit intestine. Using CsCl, ATP and cAMP in the internal solution, inward Ca^{++} channel currents could be recorded. These currents were usually small in amplitude and ran down very quickly. The addition of Cs_2-aspartate, K-pyruvate, K-succinate, phosphocreatine-Na_2 and creatine phosphokinase to the internal solution increased the amplitude by more than two fold and prolonged the time during which the Ca^{++} channel currents could be measured. Further experiments are needed to answer the question of whether the recording of Ca^{++} channel currents is related to the presence of phosphocreatine and creatine phosphokinase in the pipette solution. The presence of Cs_2-aspartate and other components in the pipette solution may contribute to the decreased "run-down" of calcium channel currents in the present study. Cs_2-aspartate may also have a significant buffer capacity for intracellular calcium although the accumulation of free calcium in the cytosol is not likely due to the use of calcium-free bath solution with high concentration of barium, and the inclusion of EGTA in the pipette solution. In addition, calcium channel currents could not be found immediately following the penetration of the cell membrane. This is due to the existence of the outward K^+ currents, which overlap the small inward Ca^{++} channel currents, as suggested in a previous study by Toro *et al.* (1986). This is perhaps the reason why Toro *et al.* (1986) could not identify the Ca^{++} currents in tail artery smooth muscle cells during their electrophysiological studies of these cells. After the cell is fully dialysed by the pipette solution and the K^+ currents are completely blocked by a high concentration of Ba^{++} in the bath solution and Cs^+ in the pipette solution, the detection of the inward currents is possible. The recently developed permeabilization method using nystatin in the pipette solution (Horn and Marty, 1988) makes it possible to record calcium channel currents without disruption of the native cytoplasmic composition. Using this method in smooth muscle cells from rat tail artery may facilitate the studies of calcium channels in this cell preparation under more physiological conditions.

As previously reported (Hess *et al.*, 1984; Bean *et al.*, 1986; Caffery *et al.*, 1986), Bay K-8644 selectively amplified the L-type calcium channel current. The multiple effects of this dihydropyridine can be described as follows: 1) It increased slowly inactivating inward currents more than two-fold. 2) This enhancement was more significant at test pulses ranging from 0 to +20 mV. 3) It stimulated the

L-type calcium channels to open at a more negative potential, i.e. when the activation threshold potential had been lowered. 4) It shifted the half-inactivation potential and half activation potential in the direction of hyperpolarization. 5) It shifted the peak of the I-V plot toward negative potentials by 10 to 20 mV. 6) It activated those calcium channels which initially were undetectable. 7) The augmentation of L-channel currents by Bay K-8644 was time-dependent. The maximum amplification occurred between 5 to 10 min after Bay K-8644 treatment and the current then declined toward the original level.

All of the above considerations point to the conclusion that calcium channels are present in rat tail artery smooth muscle cells. Since the resting potential of rat tail artery smooth muscle cells has been reported to range from -30 to -56 mV (Hermsmeyer, 1983; Bryant *et al.*, 1985), T channel currents at this membrane potential may be strongly inactivated. It is, therefore, suggested that T channels may not be important under physiological conditions. L channels would be essential in carrying inward current during action potential and providing the pathway for the extracellular calcium entry during sustained depolarization. The finding of calcium channels in rat tail artery is important because this small diameter artery, being very sensitive, is often used in pharmacological assays of vasoactive substances. The presence of calcium channels in its smooth muscle cells will provide the opportunity to study the cellular mechanisms involving regulation of calcium entry through specific voltage- and time-dependent channels. The present study is of particular importance since calcium channels could not be previously demonstrated in the vascular smooth muscle cells of the rat tail artery.

The effect of temperature on calcium channel currents in ventricular myocytes (Cavalie *et al.*, 1985) and skeletal muscle (Walsh *et al.*, 1986) has been reported. In the present study, a temperature-dependence of both the amplitude and kinetics of L channel currents in vascular smooth muscle cells are described for the first time. The Q_{10} of the amplitude of the L channel current was 2.2 ± 0.25 in the temperature range of 22° to 35°C. This compares to a Q_{10} (I_{Ca} amplitude) of 2.96 reported for ventricular myocytes (Cavalie *et al.*, 1985) and 2.3 ± 0.1 for a slow calcium current in neurones of *Lymnaea stagnalis* (Byerly *et al.*, 1984). In skeletal muscle fibers, Walsh *et al.* (1986) found that increasing the temperature from 22° to 32°C doubled the calcium current amplitude, which corresponded to a Q_{10} value of approximately 2.

Since the movement of calcium into the cell via calcium channels is involved in excitation-contraction coupling, increased inward L channel currents at higher temperatures should in turn lead to an increase in the intracellular calcium concentration and maximal contraction force. Peiper (1984), however, reported that in vascular smooth muscle from the rat portal vein the maximal contraction force was weakly temperature dependent whereas the kinetics were strongly temperature dependent with a Q_{10} of 1.8 - 2.0. The temperature dependence of calcium channels in the plasma membrane of their preparation was not known. Since the rat tail artery has characteristics similar to those of a resistance blood vessel and since the calcium required for excitation-contraction coupling in this type of smooth muscle cell comes from extracellular rather than intracellular sources, one could speculate that the maximum contraction force in this preparation would follow the temperature dependence of the inward calcium current.

Ionic channels are water-filled pores. The conductance of an open channel was believed to be relatively temperature insensitive with a Q_{10} of only 1.2 to 1.5, corresponding to an activation energy of 5 kcal/mol, which indicated a simple aqueous diffusion (Hille, 1984). A Q_{10} larger than 2 for the conductance of ionic channels would indicate that temperature-sensitive enzymatic reactions are an integral part of the conductance mechanisms. However, recent studies have revealed more complicated characteristics of the temperature sensitivity of the conductance of ion channels. At a certain range of temperatures, not only calcium channel conductance, as shown in the present study, but also sodium channel (Kimura and Meves, 1979) and potassium channel (Hagiwara and Yoshii, 1980) conductances were strongly temperature dependent with a Q_{10} larger than two. As suggested by Cavalie and co-workers (1985), the high value of Q_{10} for the conductance of calcium channels, specifically in rat tail artery smooth muscle cell L channels, could indicate that calcium channels are regulated by a cellular metabolic mechanism which is temperature dependent. It is also possible that some of the current changes (particularly those with respect to amplitude) which are reported here may not be entirely controlled by the underlying kinetics but rather by the ion transfer process as may be the case in squid axon sodium channels (Cohen and Landowne, 1974; Kimura and Meves, 1979). It should be noted that the Q_{10} is a thermodynamic concept which can only be used under completely homogenous reaction conditions (fully reversible) and does not involve any membrane delimitated reactions.

At temperatures greater than 35°C (36° to 40°C), the Q_{10} of current amplitude was 0.56 ± 0.14 with the maximum current occurring at 35°C in rat tail artery smooth muscle cells. The mechanism which may be responsible for the reduced currents at temperatures higher than 35°C remains open to speculation. In rat cardiac membrane fragments, prolonged ischemia at 37°C decreased the density of dihydropyridine binding sites, while no such change was detected during ischemia at 22°C. These results led Gu *et al.* (1988) to speculate that internalization of calcium channel proteins at higher temperatures was involved. It is also possible that the conductance of an open channel is governed by a sequence of reactions and one of these reactions may become rate limiting at 35°C (Fischbach and Lass, 1978). If calcium activated potassium channels were activated at a higher temperature, the calcium channel currents might be masked. Given the fact that the inward charge carrier is Ba^{++} and the outward charge carrier Cs^{+}, this possibility is not very likely although it cannot be completely ruled out yet. In addition, at temperatures greater than 35°C the L channels in smooth muscle cells of rat tail artery may become inactivated due to more rapid dephosphorylation. The temperature at which the maximum current occurs varies with the preparation. In vascular smooth muscle cells, the maximum current appeared at 35°C, while in neuroblastoma cells (Narahashi *et al.*, 1987) the maximum current occurred at 30°C. In ventricular myocytes (Cavalie *et al.*, 1985), the temperature dependence of the slow inward calcium channel current was continuous from 21° to 37°C. These differences may be due to the different temperature sensitivities of various tissues as related to their physiological functions.

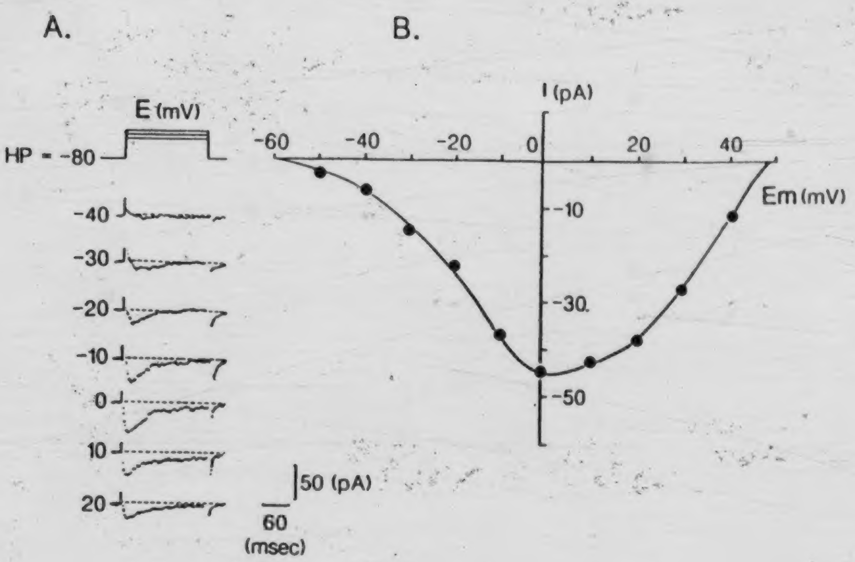

A.
B.
E (mV)
HP = -80
-40
-30
-20
-10
0
10
20
50 (pA)
60
(msec)
I (pA)
-60
-40
-20
0
20
40
-10
-30
-50
Em (mV)

Fig. IV-1. T-type barium currents in one vascular smooth muscle cell. Some L-type current is also present. This cell was cultured for 22 hr after isolation. **A.** Original current records were recorded 8 min after the cell was voltage clamped. The holding potential (HP) was -80 mV, and test pulses are denoted beside each record. Zero current levels are designated by dotted lines. Leakage currents have been subtracted. Capacitive currents have also been subtracted but subtraction was imperfect. **B.** Current-voltage relationship for a single cell. Peak inward current occurred at 0 mV and the inward current reversed at +50 mV. I, current; E, potential; Em, membrane potential.

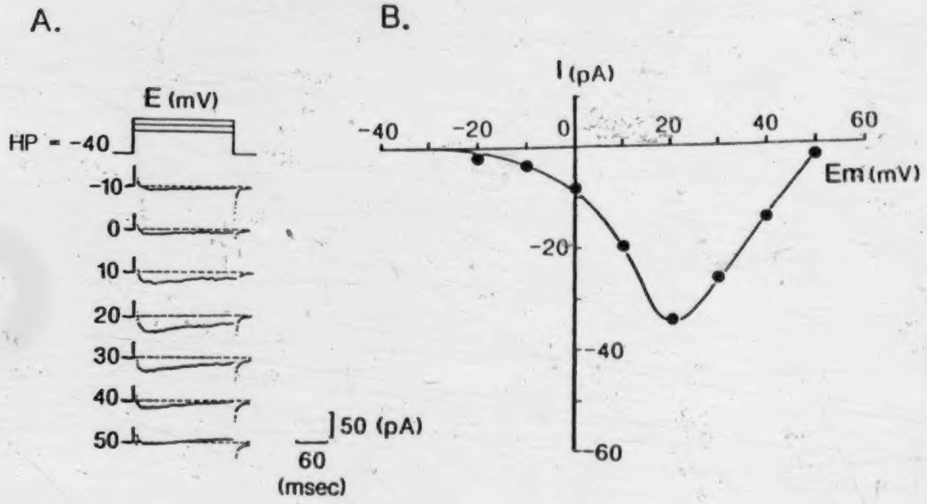

A.
E (mV)
HP = -40
-10
0
10
20
30
40
50
50 (pA)
60
(msec)
B.
I (pA)
-40
-20
0
20
40
60
Em (mV)
-20
-40
-60

Fig. IV-2. L-type barium currents in one vascular smooth muscle cell cultured for 24 hr after isolation. **A.** Current records were obtained 5 min after the cell was voltage clamped. Holding potential was -40 mV, and test pulses are denoted beside each record. Zero current levels are designated by dotted lines. As in Fig. IV-1, leakage and capacitive currents (incomplete) are subtracted. **B.** Current-voltage relationship for a single cell. The L-type current was activated at -20 mV with a peak inward current at +20 mV. This current reversed at +50 mV. 20 mM Ba^{++} was used in the bath solution. See Fig. IV-1 for abbreviations.

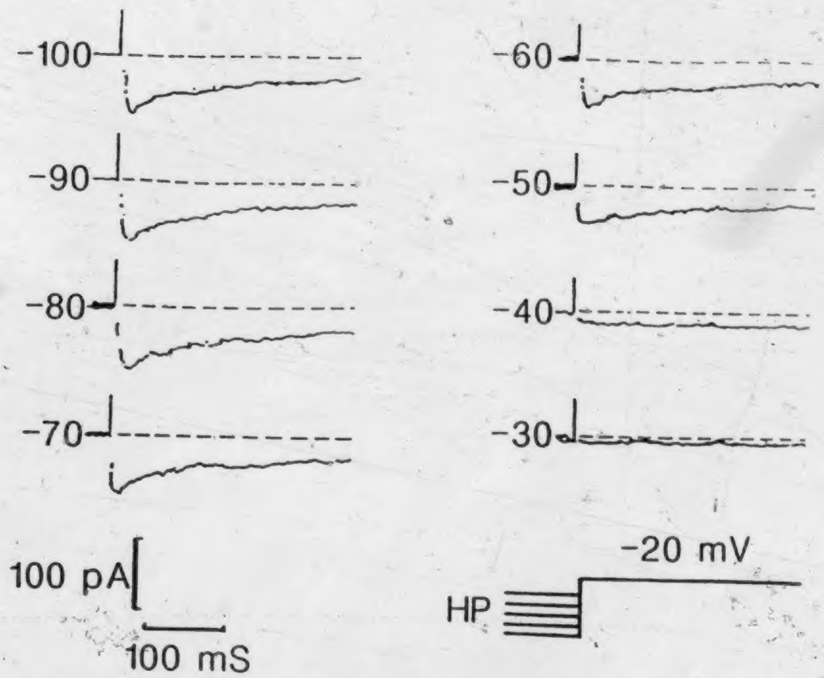

-100
-90
-80
-70
-60
-50
-40
-30
100 pA
100 mS
-20 mV
HP

Fig. IV-3. Separation of two types of calcium channel currents in one vascular smooth muscle cell. Barium currents were evoked using a test pulse to -20 mV from various holding potentials (HP). Test pulses from holding potentials varying from -100 mV to -60 mV evoked both T- and L-type calcium channel currents. T channel currents were inactivated when holding potentials became more positive than -50 mV while L channel currents could still be detected. L channel currents were measured as the current values at 200 msec from the start of the test pulse. Dashed lines are the zero current levels. Records are leakage corrected.

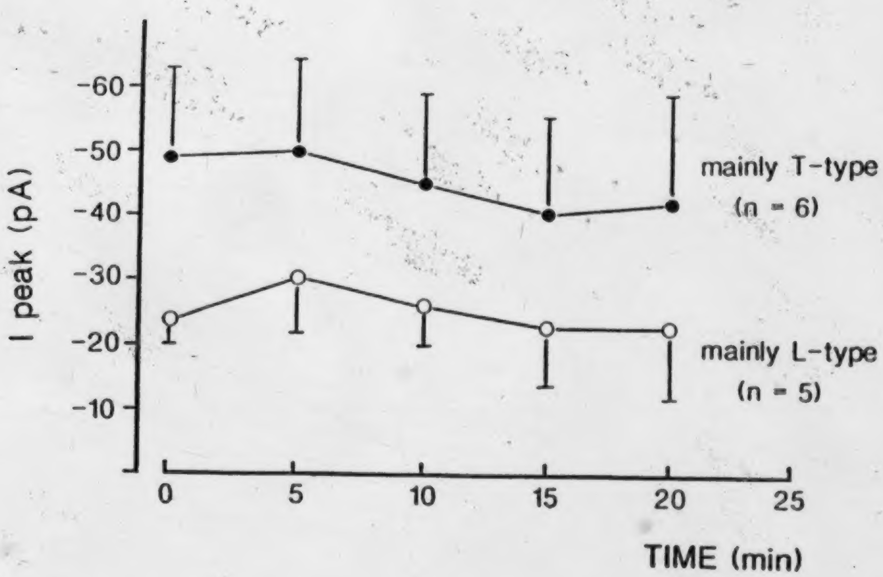

I peak (pA)
-60
-50
-40
-30
-20
-10
0
5
10
15
20
25
TIME (min)
mainly T-type
(n = 6)
mainly L-type
(n = 5)

Fig. IV-4. Stability of calcium channel currents in primary cultured vascular smooth muscle cells. Time zero was taken when inward currents emerged (1 or 3 min after penetration of cell membrane). Currents (I) peaked at about 5 min. This was the case for both T-type and L-type calcium channels. Each point represents the mean and vertical bars represent SE. The L-channel component was not subtracted from predominantly T-type calcium channel currents.

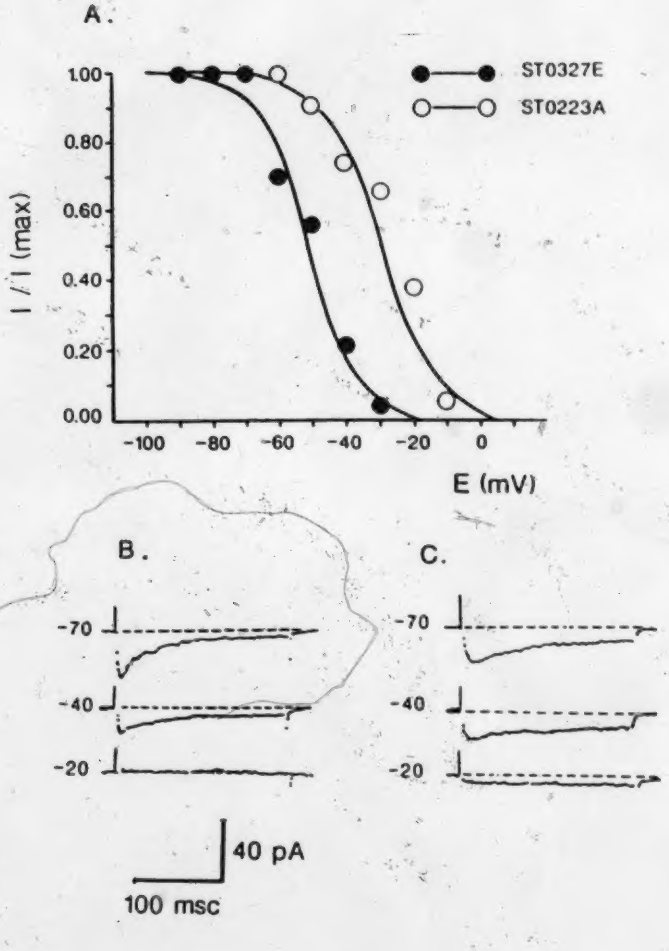

A.
1.00
0.80
0.60
0.40
0.20
0.00
I / I (max)
ST0327E
ST0223A
-100
-80
-60
-40
-20
0
E (mV)
B.
C.
-70
-40
-20
-70
-40
-20
40 pA
100 msc

Fig. IV-5. Steady-state inactivation of T- and L-type calcium channels in vascular smooth muscle cells. **A.** Steady-state inactivation of L-type calcium channels in one cell (open circles); steady-state inactivation of T-type calcium channels in another cell (filled circles). Continuous curves were fitted by the equation $I = I_{max}[1 + \exp(V - V_h)/k]^{-1}$. For the cell with predominantly T channel currents, V_h = -50.5 mV and k = 6.9 mV. In the cell with predominantly L channel currents, V_h = -28.6 mV and k = 8.2 mV. **B.** Current records used to generate the steady-state inactivation of T channel currents (filled circle). **C.** Currents used to generate the steady-state inactivation of the L-channel current (open circles) in a cell with predominantly L channel currents. In both **B** and **C** peak current values were used.

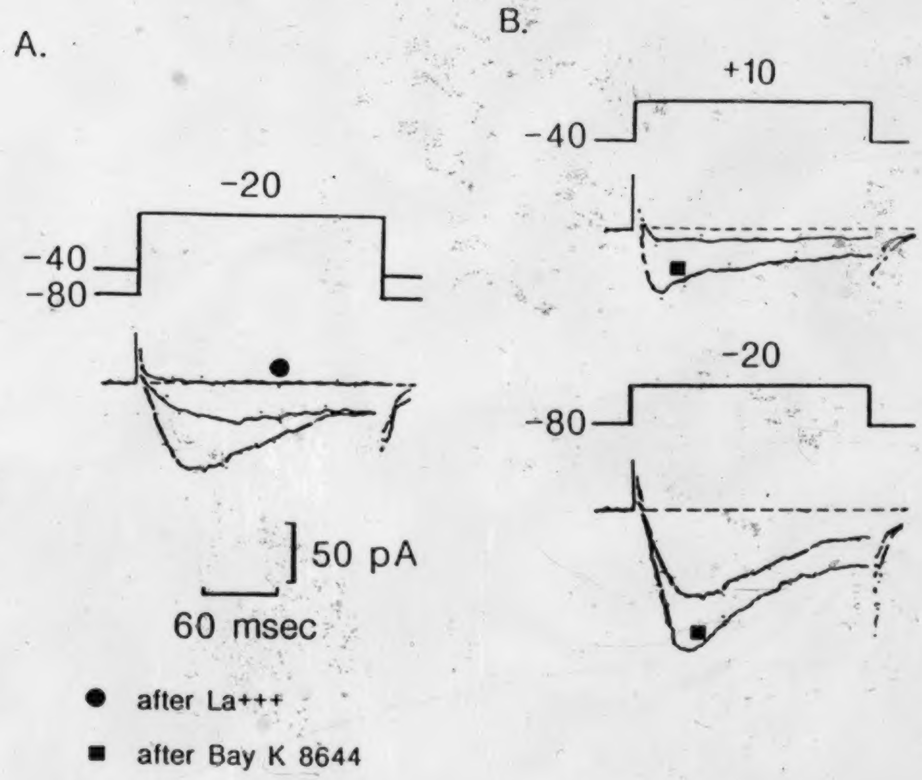

A.
B.
+10
-40
-20
-40
-80
-20
-80
50 pA
60 msec
● after La+++
■ after Bay K 8644

Fig. IV-6. The sensitivity of two types of calcium channel currents to La^{+++} and Bay K-8644 in vascular smooth muscle cells. Current records are shown below test pulses. Current records are corrected for leakage and capacitive currents. **A.** The bottom current record was obtained by depolarizing the cell membrane to -20 mV. In this cell, both T- and L-type calcium channels coexisted. The middle current record showed only L-type calcium channel activity when the holding potential was set to -40 mV. The upper current record was recorded 2 min after the addition of La^{+++} (2 mM final concentration). Cell: ST0327A. **B.** Modulation of calcium channel currents by Bay K-8644 (5 μM final concentration). The upper current records show (holding potential -40 mV) that Bay K-8644 increased L-type calcium channel currents by a factor of 4.6. Cell: ST0226C. In the lower current records (holding potential -80 mV) Bay K-8644 increased the inward current in a cell with predominantly T-channels by about 60 %, but this increase was due to the effect of Bay K-8644 on the L-channel component. Cell: ST0327A.

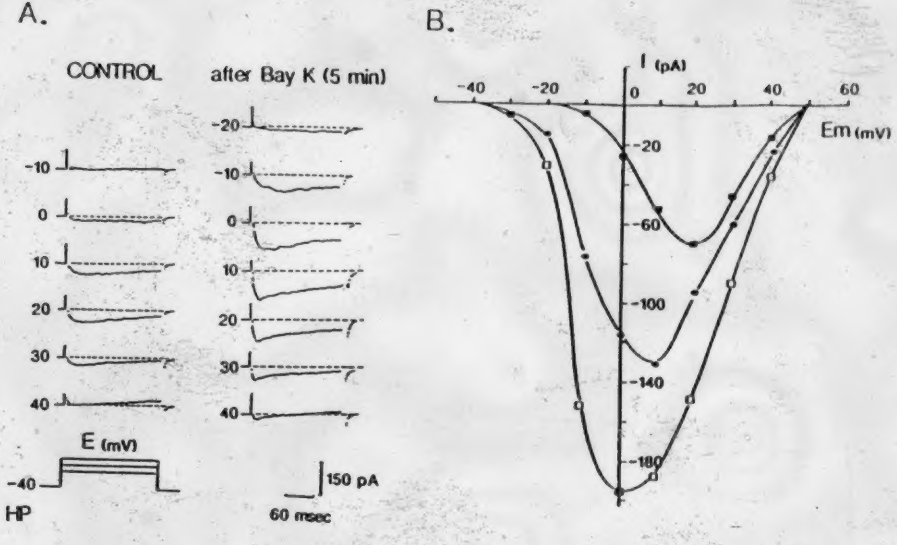

A.
CONTROL
after Bay K (5 min)
-20
-10
0
10
20
30
40
E (mV)
-40
HP
150 pA
60 msec
B.
I (pA)
-40
-20
0
20
40
60
Em (mV)
-20
-60
-100
-140
-180

Fig. IV-7. Modulation of L-type calcium channel currents by Bay K-8644 in vascular smooth muscle cells. **A.** Inward currents obtained before and after addition of Bay K-8644 (final concentration of 5 μM). Holding potential (HP) was -40 mV. Test pulses are indicated beside each trace (mV). **B.** Current voltage relationships obtained before (filled squares), 5 min (filled circles) and 10 min (open squares), respectively, after treatment with Bay K-8644.

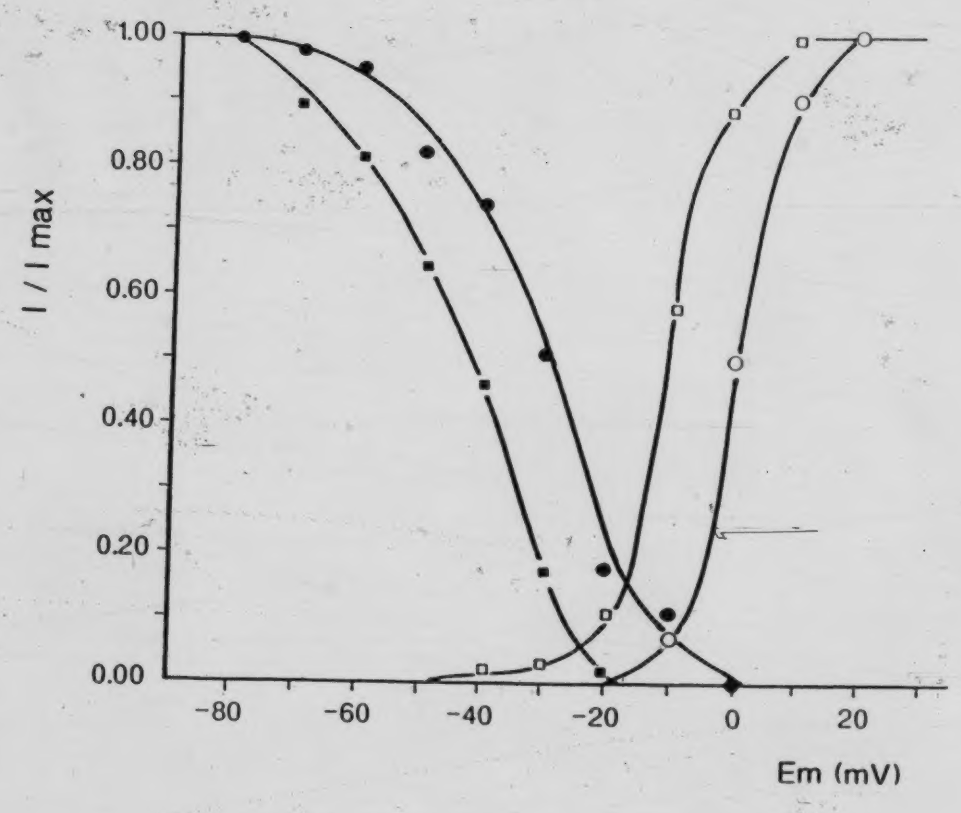

1.00
0.80
0.60
0.40
0.20
0.00
I / I max
-80
-60
-40
-20
0
20
Em (mV)

Fig. IV-8. Bay K-8644 shifted steady-state inactivation and activation curves of L channels in vascular smooth muscle cells. The filled circles and squares indicate the steady-state inactivation curves in the absence and then presence of Bay K-8644, respectively. The open circles and squares represent the steady-state activation curve in the absence and then presence of Bay K-8644, respectively. The final concentration of Bay K-8644 was 5 μM. Solid curves represent data fitted to equations of the forms $I = I_{max}[1 + \exp(V - V_h)/k]^{-1}$ (inactivation) and $I = I_{max}[1 + \exp(V_h - V)/k]^{-1}$ (activation).

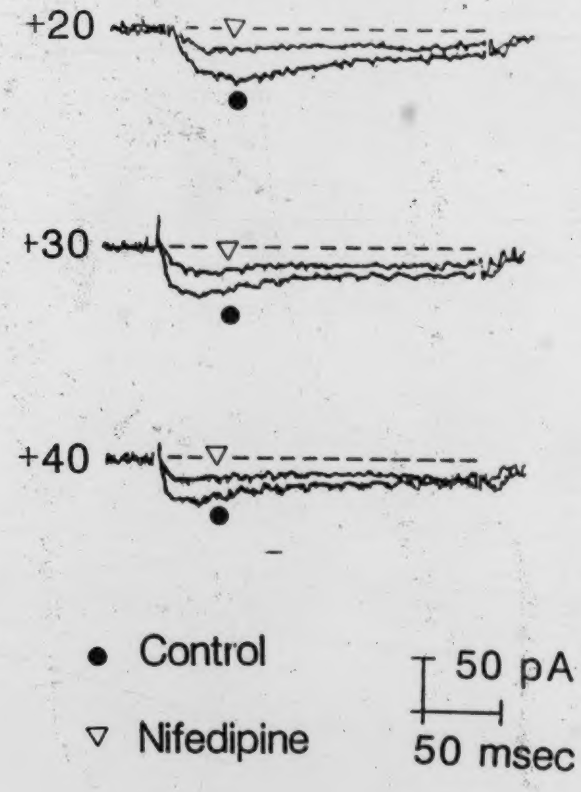

+20
+30
+40
● Control
▽ Nifedipine
50 pA
50 msec

Fig. IV-9. The inhibition of L channel currents in one vascular smooth muscle cell by nifedipine (0.5 μM). The holding potential was set at -40 mV. Test pulses are indicated beside each current trace. Dashed lines represent zero current level. Leakage currents and capacitive transients have been corrected from original traces.

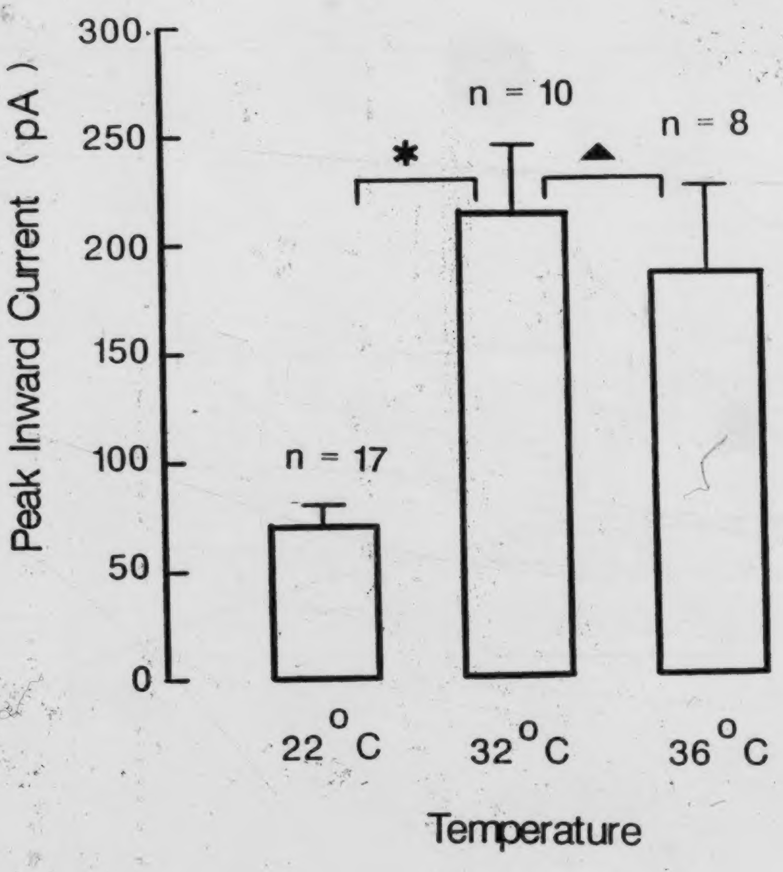

Peak Inward Current (pA)
300
250
200
150
100
50
0
n = 17
n = 10
n = 8
*
▲
22 °C
32 °C
36 °C
Temperature

Fig. IV-10. The amplitude of L channel currents in vascular smooth muscle cells at three different temperatures. * indicates a significant difference between the groups compared ($p<0.05$), while ▲ indicates differences which were not significant ($p>0.05$). The number of cells in each group has been indicated on the figure.

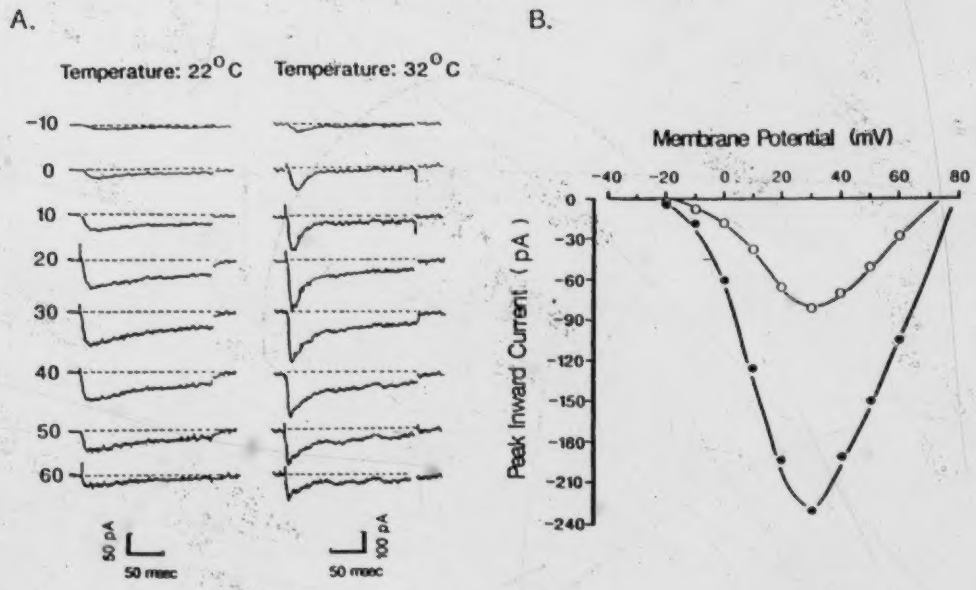

A.
B.
Temperature: 22°C
Temperature: 32°C
-10
0
10
20
30
40
50
60
50 pA
50 msec
100 pA
50 msec
Membrane Potential (mV)
-40
-20
0
20
40
60
80
Peak Inward Current (pA)
0
-30
-60
-90
-120
-150
-180
-210
-240

Fig. IV-11. Effect of temperature on L channel currents in vascular smooth muscle cells. **A.** Inward currents were elicited from a holding potential of -40 mV at two temperatures (22°C and 32°C) in the same cell. All current traces were corrected for capacitive and leak currents. The test potentials are marked beside each trace. Note the difference in the calibration of the current amplitudes at 22°C and 32°C. **B.** The current-voltage (I-V) relationships of L channels at 22°C and 32°C are taken from the same cell as in **A.**

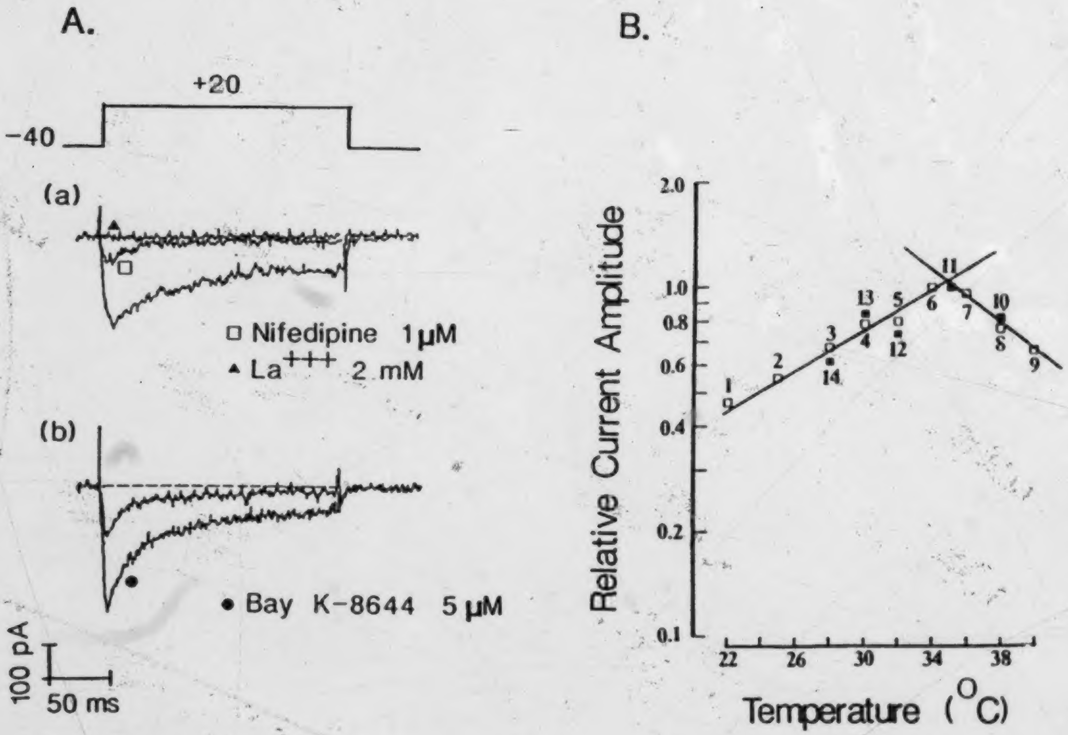

A.
+20
-40
(a)
□ Nifedipine 1 μM
▲ La+++ 2 mM
(b)
● Bay K-8644 5 μM
100 pA
50 ms
B.
Relative Current Amplitude
2.0
1.0
0.8
0.6
0.4
0.2
0.1
22
26
30
34
38
Temperature (°C)
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig. IV-12. Effects of dihydropyridines on L channel currents in vascular smooth muscle cells at various temperatures. **A.** The effect of dihydropyridines on L channel currents at 32°C. The currents were recorded from two different cells, shown in (a) and (b), with the test potential to +20 mV from a holding potential of -40 mV. The control traces were shown as the lowest one in (a) and the top one in (b). 1 μM nifedipine was followed by 2 mM La^{+++} which completely blocked the inward current in (a). 5 μM Bay K-8644 increased the inward currents by twofold in (b). The capacitive and leakage currents were subtracted in (a) and (b). The dashed line represents the zero current level in (b). **B.** Logarithm of the relative amplitude of L-type Ca^{++}-channel currents as a function of temperature. The inward currents were recorded at a test potential of +10 mV from a holding potential of -40 mV. The number attached to each data point indicates the sequence of recording. The open squares represent the steps of increasing temperature and the filled squares the steps of decreasing temperature. The straight lines are fitted by eye.

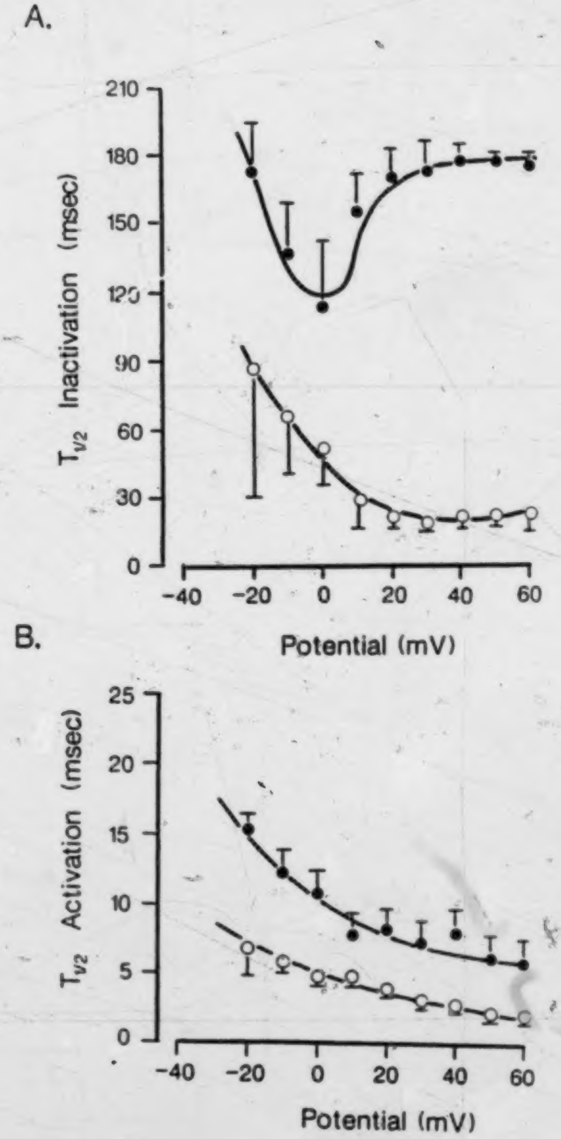

A.
210
180
150
120
90
60
30
0
$T_{1/2}$ Inactivation (msec)
-40
-20
0
20
40
60
Potential (mV)
B.
25
20
15
10
5
0
$T_{1/2}$ Activation (msec)
-40
-20
0
20
40
60
Potential (mV)

Fig. IV/13. Temperature- and potential-dependent activation and inactivation of L channel currents in vascular smooth muscle cells. In **A** and **B** data points are mean values of 6 (filled circles) or 7 (open circles) cells, and vertical bars represent the standard errors. The peak amplitude of L channel currents was measured at +10 mV from a holding potential of -40 mV. Filled circles represent the value measured at 23°C and open circles at 36°C. The smooth curves are fitted by eye.

CHAPTER 5

Characteristics of voltage-dependent calcium channels in neonatal rat ventricular myocytes

INTRODUCTION

Using the whole-cell or single channel recording technique, two types of voltage-dependent calcium channels have been identified in guinea pig ventricular, dog atrial, frog atrial and rabbit sinoatrial cells (Bean, 1989a, b). Different criteria or a combination of criteria have been employed to separate the various types of cardiac calcium channels. Variation of holding potentials can handily distinguish T and L channels from each other. The kinetics of various types of calcium channel currents is also different. It is well known that the inactivation of calcium channel currents has a joint dependence: it is both calcium- and voltage-dependent (Lee *et al.*, 1985). When Ba^{++} or Sr^{++} is used as the inward charge carrier, the inactivation of L channel currents is significantly reduced in guinea pig ventricular cells (Hess *et al.*, 1986; Hadley and Hume, 1987), rat ventricular myocytes (Mitchell *et al.*, 1983; Josephson *et al.*, 1984), and canine atrial myocytes (Bean, 1985). The inactivation kinetics of T channel currents, however, remains the same with Ba^{++} or Ca^{++} as the charge carrier. A higher sensitivity of L channels to dihydropyridines, and T channels to nickel and tetramethrin (Hagiwara *et al.*, 1988) has made the pharmacological dissection of L and T channel currents feasible. A detailed description of the characteristics of cardiac calcium channels can be found in several recent reviews (Bean, 1989a, b; Hess, 1990; Pelzer *et al.*, 1990).

In addition to the above electrophysiological and pharmacological characteristics of cardiac T and L channels, the distribution, characteristics and functional importance of calcium channels are also different during different developmental stages of cardiac myocytes. Although the existence of L channels in neonatal rat ventricular cells has been established (Tsien *et al.*, 1986), typical T channels have not been fully described or characterized except for a brief description by Rampe *et al.* (1989). Cohen and Lederer (1987, 1988) characterized L channel currents in neonatal rat ventricular cells. Their research concentrated on L channel currents and the holding potential was set at -50 mV. T channel currents were not studied in this report.

The activation of L channels is mainly responsible for extracellular calcium entry upon depolarization. L channels and sarcoplasmic reticulum compose the fundamental link in excitation-contraction coupling in adult rat ventricular myocytes (Cohen and Lederer, 1988). However, in neonatal rat ventricular cells, L channels are more important in triggering cell contraction since the sarcoplasmic reticulum is poorly developed at a very early stage (Cohen and Lederer, 1988; Kilborn and Fedida, 1990). The absence of a functional sarcoplasmic reticulum has also been demonstrated in immature heart cells (Boucek *et al.*, 1985). T channels, on the other hand, have been related to the electrical excitability of cardiac myocytes. Mainly distributed in atrial cells (Bean, 1989a, b), T channels contribute very little to the total calcium channel currents in ventricular cells (Nilius *et al.*, 1985).

When a comparison was made between adult and neonatal rat ventricular myocyte, it was found that L channels had a higher density and slower inactivation kinetics in neonatal rat ventricular cells than in adult ones (Cohen and Lederer, 1988). The calcium-dependent mechanism has been suggested to be partially responsible for the more rapid inactivation of L channel currents in adult rat ventricular cells (Cohen and Lederer, 1988). However, in neonatal rat ventricular cells, the changes in intracellular calcium concentration failed to correlate with inactivation of L channels (Cohen and Lederer, 1987, 1988).

In the experiment described in this chapter, two types of calcium channels are characterized in neonatal rat ventricular myocytes. The characterization of T and L channels in neonatal rat ventricular myocytes will help to elucidate the developmental changes of different types of calcium channels and their developmental roles. On the basis of this characterization of calcium channels it will become possible to investigate further the effects of PTH and other agents on different types of calcium channels in this cell preparation.

RESULTS

Electrophysiological characteristics.

The current recording was carried out in spherical ventricular myocytes. With 20 mM Ba^{++} as the charge carrier, the inward current elicited from a holding potential of -80 mV to various test pulses showed two basic patterns in terms of magnitude and kinetics. At relatively negative potentials, the current inactivated quickly and completely. As shown in Fig. V-1, these currents, transient in appearance, reached the maximum amplitude at membrane potentials around -20 mV and inactivated quickly. At potentials more positive than -30 mV, the inward current inactivated slowly during a 200 msec pulse. The maximum amplitude of this slowly inactivating current occurred at membrane potentials around +10 mV. Simultaneously, the transient component decreased its amplitude. It has been reported that in almost all adult cardiac cell types examined to date the maximal magnitude of T channel currents is typically only 5-20% that of L channel currents. T channel currents were not detectable in some ventricular cell types (Bean, 1989a). However, in neonatal rat ventricular cells, T channel currents with variable magnitudes, ranging from -50 to -200 pA were found. In several cells, the amplitude of T channel currents was greater than that of L channel currents in the same cell. In contrast, in some cells, only L channel currents could be recorded. In those cells which exhibited both T and L channel currents, the shift of holding potential from -80 mV to -40 mV inactivated T channel currents but had little effect on L channel currents. As shown in Fig. V-2, the amplitude of L channel currents was similar at a holding potential of -80 mV or -40 mV. This result was in contrast to that in vascular smooth muscle cells in which L channel currents were half inactivated at membrane potentials around -30 mV (Chapter 4). Hence, the steady-state inactivation of L channel currents was studied in neonatal rat ventricular myocytes. The membrane potential was held at various levels between -80 to +10 mV and 200 ms depolarizing test pulses were applied to +20 mV (Fig. V-3). The peak amplitudes of L channel currents were normalized and plotted against holding potentials. A sigmoidal shape of steady-state inactivation of L channel currents was revealed. Up to a holding potential of -40 mV, no inactivation of L channel currents was found. Half inactivation of L channel currents occurred at approximately -5 mV. The normalized conductance curve of L channels is shown in Fig. V-5. The conductance was calculated from the equation: $G_{(L)} = I_{(L)} / (V_T - V_{rev})$. The apparent reversal potential of L channels is determined as the intersection of the I-V curves obtained before and after the application of 2 mM La^{+++} in the bath. V_T is the test pulse at which the L channel current, $I_{(L)}$, is recorded. The maximum conductance is taken as 1.00.

It was previously reported that T channel currents in rabbit sino-atrial node cells inactivated by 50% at holding potentials between -80 to -70 mV (Hagiwara *et al.*, 1988). In the present study, the steady-state inactivation of T channel currents in neonatal rat ventricular myocytes were not studied because of a variable population of T channel currents and the difficulty in separating T from L channel currents.

Pharmacological characteristics.

Although L channel currents were abolished by the addition of 1 μM nifedipine, the T channel current remained (see Chapter 8). Consistent with other studies of rat ventricular cells (Sanguinetti *et al.*, 1986), Bay K-8644 potentiated L channel currents in the present studies. The relative amplitudes of L channel currents in the absence or presence of 5 μM Bay K-8644 were 100% and 302 ±48%, respectively ($n=7$, $p<0.05$). Further analysis showed that Bay K-8644 also increased the rate of inactivation of L channel currents (Fig. V-4). The I-V relationship of L channel currents was shifted toward more negative potentials by more than 10 mV in the presence of Bay K-8644 (Fig. V-4), as were the normalized conductance curves (Fig. V-5). These modifications of L channel currents by Bay K-8644 are similar to those found previously in vascular smooth muscle cells (Wang *et al.*, 1989), but different from those in neuroblastoma cells (Wang *et al.*, 1988, 1990).

According to the above electrophysiological and pharmacological characteristics of the two types of inward currents in neonatal rat ventricular cells, the transient current was identified as the T channel current and the sustained current as the L channel current.

DISCUSSION

The action potential of many mammalian ventricular tissues has an elevated plateau phase and lasts more than 100 ms (Cavalie et al., 1983; Giles and Brown, 1983; Agus et al., 1989; White and Terrar, 1990). The adult rat ventricular action potential lasts less than 100 ms without a recognisable plateau phase (Cohen and Lederer, 1988; Kilborn and Fedida, 1990). However, in the early stage of development, the action potential of rat ventricular myocytes is very similar to other mammalian ventricular muscle preparations in exhibiting a long-lasting and elevated plateau phase (Cohen and Lederer, 1989; Kilborn and Fedida, 1990). The changes in calcium currents (Van Ginneken and Jongsma, 1983; Brown *et al.*, 1986; Cohen and Lederer, 1988) and potassium currents (Kilborn and Fedida, 1990) are believed to be determining factors in the developmental changes in rat ventricular muscle action potential. The similarity of action potentials with those of other mammalian ventricular myocytes, the larger density of calcium channels compared to adult myocytes; and the easy with which single cells may be isolated make neonatal rat ventricular cells a suitable model to investigate the distribution, function and modulation of different types of calcium channels.

Two types of voltage- and time-dependent calcium channel currents in neonatal rat ventricular cells have been identified and described here. The transient calcium channel (T channel) current activated quickly and inactivated completely within the 200 msec depolarization. On the other hand, the long-lasting calcium channel (L channel) current was activated at more positive membrane potentials and inactivated very slowly. L channel currents were sensitive to dihydropyridines whereas T channel currents were not. The characteristics of L and T channel currents in neonatal rat ventricular myocytes are similar to those in canine atrial cells (Nilius *et al.*, 1985). L channel currents were, however, predominant. T channel currents in most of the cells tested had an amplitude of 50 to 200 pA, which was consistent with the T channel amplitude in other studies. For example, Rampe and co-workers (1989) reported amplitudes of 25 to 60 pA. In order to eliminate the possibility that the T channel current recorded in rat myocytes was contaminated by sodium channel currents, a Na^+-free extracellular solution was used. In addition, a relatively high concentration of TTX was also included in the extracellular solution. Furthermore, the inactivation of T channel currents in neonatal rat ventricular myocytes was much slower than that of sodium channel currents.

One obstacle to the study of voltage- and time-dependent calcium channels in neonatal rat ventricular cells is the fast run-down or deterioration of the inward currents (Yatani *et al.*, 1986). Several guidelines have been set up in the present study to overcome this difficulty. First, an ATP-regenerating system was included in the pipette solution to delay the run-down. Second, the control level of currents was not established until there was no change in the current amplitude for 5 min. Third, careful comparison was made between the records obtained with and without the tested agents in the bath solution, using the same time scale. Fourth, the responses of inward currents to the agent tested were reversed by subsequent application of an agonist or antagonist, correspondingly.

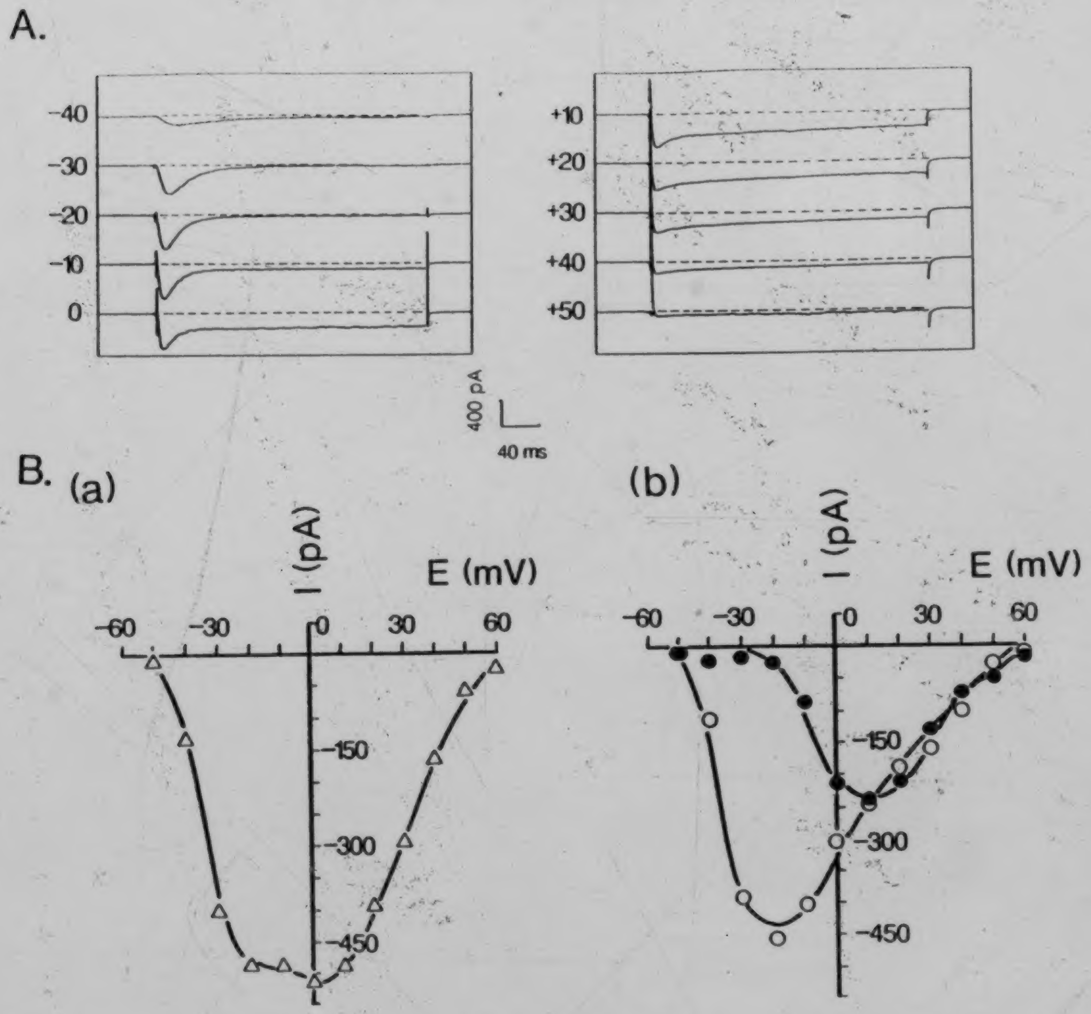

A.
-40
-30
-20
-10
0
+10
+20
+30
+40
+50
400 pA
40 ms
B.
(a)
(b)
I (pA)
E (mV)
-60
-30
0
30
60
-150
-300
-450

Fig. V-1. Two types of voltage- and time-dependent calcium channels in neonatal rat ventricular cells. The data shown in **A** and **B** were taken from the same cell with the membrane potential fixed at -80 mV. The leakage and capacitive currents have been subtracted from original current traces. **A.** The current traces were recorded at different test depolarizations, which are indicated beside each current trace (mV). The dashed lines represent the baseline. **B.** The I-V relationship of total currents (open triangle) determined as the peak amplitude of inward currents is shown in (a). A shoulder at approximately -20 mV in the I-V plot is the result of the coexistence of both T and L channel currents. Separated T and L channel currents are shown in (b). L channel currents (filled circles), determined at the end of a 200 msec test pulse, were maximal at a membrane potential of +10 mV, whereas T channel currents (open circles), determined as the difference of total currents and L channel currents, were maximal at -20 mV.

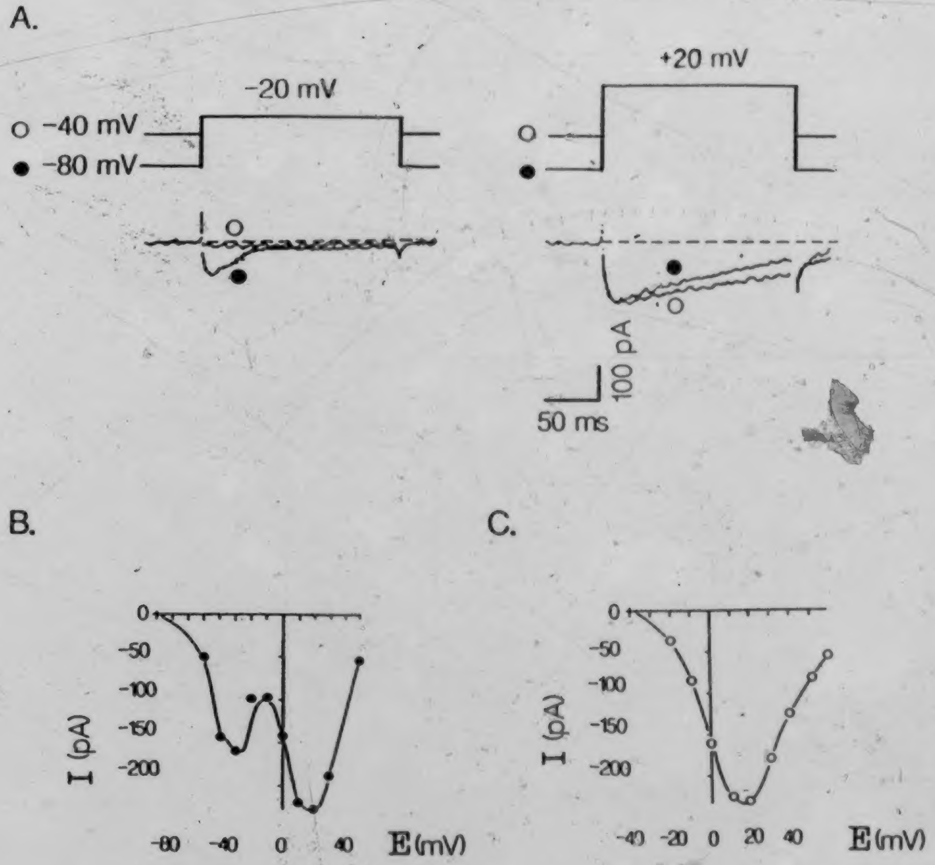

A.
-20 mV
o -40 mV
● -80 mV
+20 mV
o
●
100 pA
50 ms
B.
0
-50
-100
-150
-200
I (pA)
-80
-40
0
40
E(mV)
C.
0
-50
-100
-150
-200
I (pA)
-40
-20
0
20
40
E(mV)

Fig. V-2. The separation of two types of calcium channel currents by varying the holding potential in one ventricular myocyte. A. The original current traces at a holding potential of -80 or -40 mV. At a holding potential of -80 mV, a test pulse of -20 mV elicited predominantly T channel currents whereas +20 mV elicited predominantly L channel currents. At a holding potential of -40 mV, a test pulse of -20 mV failed to induce an inward current but +20 mV elicited predominantly L channel currents. Leakage and capacitive currents have been subtracted (see Chapter 2). Dashed lines represent the baseline. B. The I-V relationship of inward currents elicited from a holding potential of -80 mV. Two peaks on the I-V curve, -30 and +20 mV, were shown. The peak at -30 mV represented the activity of T channels and that at +20 mV, L channels. C. The I-V relationship of inward currents elicited from a holding potential of -40 mV in the same cell. Only one peak was detectable on the I-V curve, which represented L channel activity. T channel currents were inactivated.

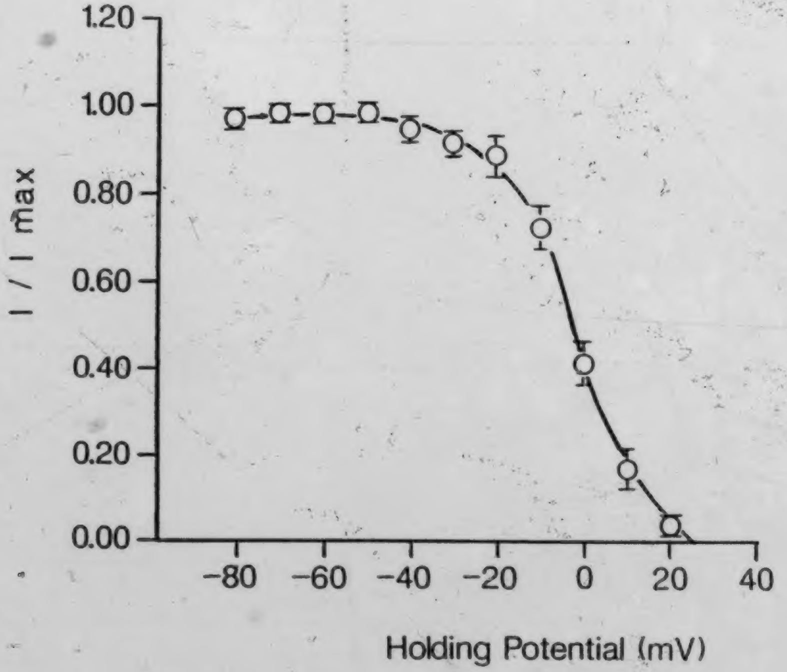

1.20
1.00
0.80
0.60
0.40
0.20
0.00
I / I max
-80
-60
-40
-20
0
20
40
Holding Potential (mV)

Fig. V-3. The steady-state inactivation of L channel currents in neonatal rat ventricular cells. The holding potential was changed in a stepwise manner with a fixed depolarizing pulse to +20 mV. The membrane potential was first conditioned for 3 sec to reach a quasi-steady state and a test pulse of 320 ms then followed. The peak current amplitudes during the test pulses were measured at each holding potential and normalized to the maximum peak amplitude of inward currents. The solid curve was fitted by eye. Each point on the sigmoidal curve represents 7 cells.

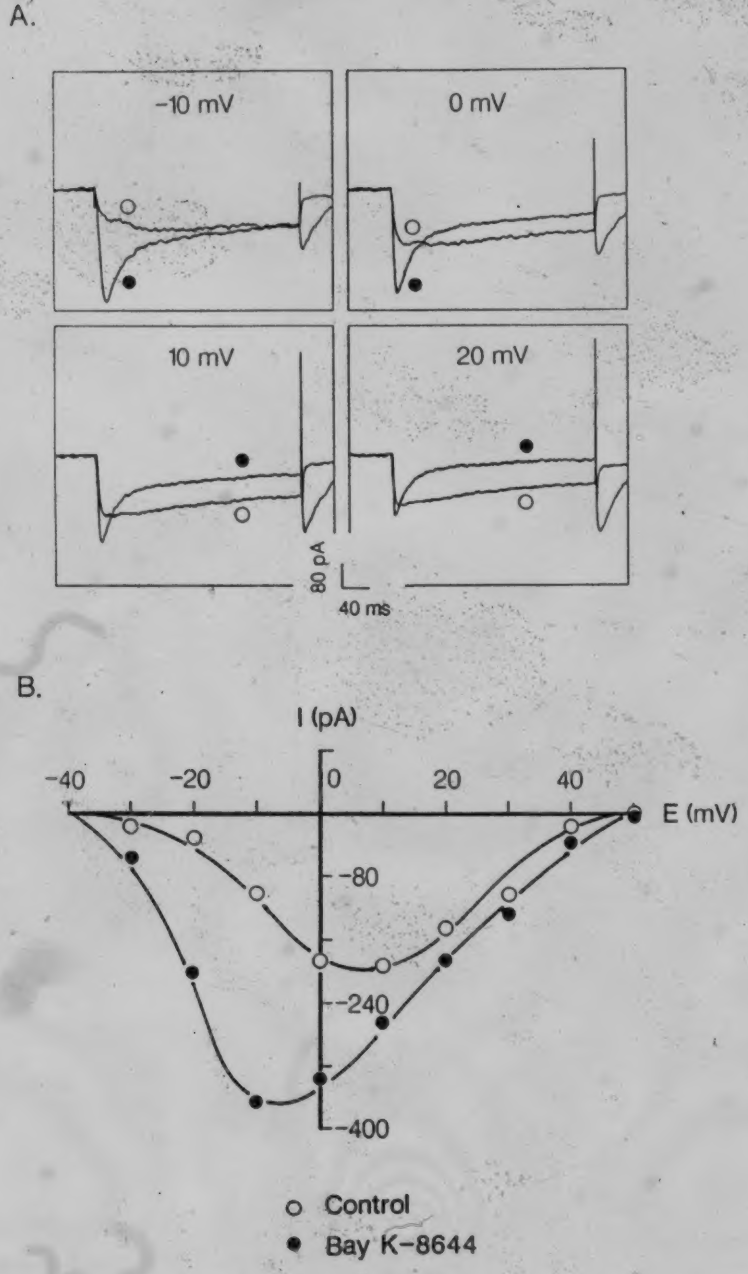

A.
-10 mV
0 mV
10 mV
20 mV
80 pA
40 ms
B.
I (pA)
-40
-20
0
20
40
E (mV)
-80
-240
-400
○ Control
● Bay K-8644

Fig. V-4. Effects of Bay K-8644 on L channel currents in a ventricular myocyte. **A.** The original current traces in the absence and then presence of 5 μM Bay K-8644. The amplitude and kinetics of L channel currents as well as the tail current were increased by Bay K-8644. Holding potential was set at -40 mV. Test pulses are indicated on the top of each set of current traces. **B.** I-V relationship of L channel currents in the absence and then presence of Bay K-8644. The peak of the I-V relationship of L channel currents was shifted toward more negative potentials by 20 mV in the presence of Bay K-8644. Leakage and capacitive currents have been corrected.

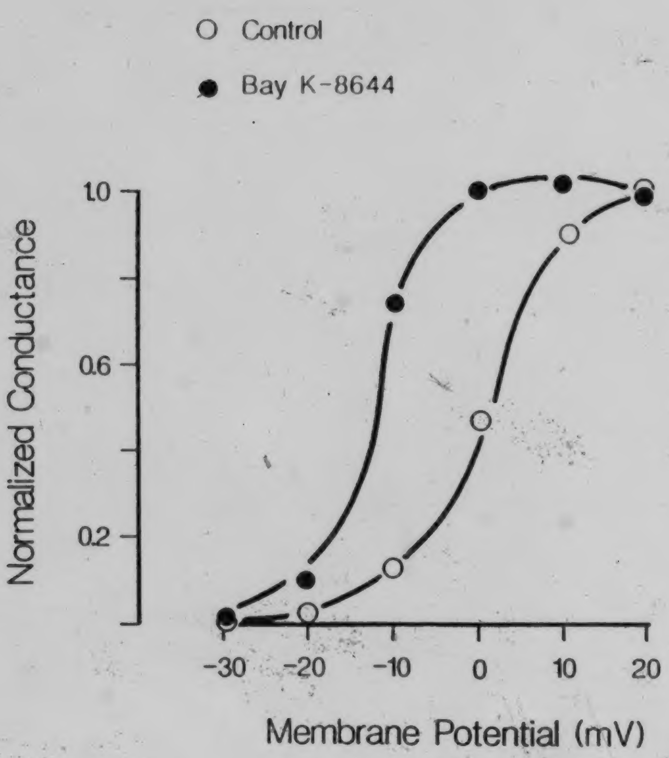

○ Control
● Bay K-8644
1.0
0.6
0.2
Normalized Conductance
-30
-20
-10
0
10
20
Membrane Potential (mV)

Fig. V-5. The normalized conductance curves of L channels in one ventricular myocyte in the absence (open circles) and then presence (filled circles) of Bay K-8644. In this cell, holding potential was set at -40 mV. The normalized conductance curve was plotted as relative conductance of L channels against membrane potentials. The maximum and half conductance of L channels under control conditions occurred at membrane potentials of approximately +20 and +5 mV, respectively. The normalized conductance curve moved toward the direction of hyperpolarization by 20 mV in the presence of 5 μM Bay K-8644. Solid curves are fitted by eye.

CHAPTER 6

The selective inhibition of L channel currents in neuroblastoma cells by bPTH-(1-34)

INTRODUCTION

The hypercalcemic effect of PTH has been well studied and firmly established. The other effects, such as vasodilation and positive inotropic action, of PTH have also been extensively investigated. However, the mechanisms underlying the cardiovascular effects of PTH are not clear. The modification of calcium channel currents by PTH might be responsible for PTH-induced cardiovascular actions, as suggested by Pang *et al.* (1988a). Instead of beginning to study the effect of PTH on calcium channel currents in vascular smooth muscle cells and ventricular myocytes, my initial experiments used neuroblastoma cells (N1E-115). There are several reasons for this choice. First, two types of calcium channels in N1E-115 cells have been successfully identified, as described in Chapter 3. Of primary importance is the controlled expression of either predominantly T channel currents or predominantly L channel currents through different culture methods. These relatively pure forms of T or L channel currents are not available in vascular smooth muscle cells or ventricular myocytes, even though these two types of channel currents have similar characteristics in both these cell preparations. Second, N1E-115 cells are readily available and are easily maintained. Third, although the function of PTH in neurons is not known, the distribution of PTH-like immunoreactivity in the CNS has been demonstrated (Pang *et al.*, 1988b, c). Hence, the study of the effect of PTH on calcium channel currents in neuronal cells can be justified.

RESULTS

The T channel current was unaffected by bPTH-(1-34).

After stable calcium channel currents were established, bPTH-(1-34) was added to the bath solution. The peak inward currents at different depolarization pulses, the relative peak current amplitudes (Fig. VI-1, 2) and I-V relationships (Fig. VI-3) of T channels were unaffected by bPTH-(1-34). The peak amplitude of T channel currents was -181±34 pA and -170±37 pA before and 5 min after the addition of 1 μM bPTH-(1-34), respectively ($n=8$, $p>0.05$). In the same group of cells, the application of 5 μM Bay K-8644 following bPTH-(1-34) did not enhance the T channel currents ($I_{peak} = -177 \pm 36$ pA, $p > 0.05$).

The L channel current was modified by bPTH-(1-34).

Fig. VI-2 shows that bPTH-(1-34) at the concentration of 1 μM decreased L channel currents to 71% of the control value ($n=9$, $p<0.05$). The I-V relationship of L channels was not affected by bPTH-(1-34) (Fig. VI-4). Subsequent application of Bay K-8644 increased the L channel currents which had previously been inhibited by bPTH-(1-34) (Fig VI-1). After the addition of 5 μM Bay K-8644, the relative peak amplitude of L channel currents was increased to 129%, when the pre-PTH value was taken as 100% (Fig. VI-2, $p<0.05$). However, there is no significant difference between the relative peak amplitude of L channel currents before the application of bPTH-(1-34) and after the application of Bay K-8644, which suggested that bPTH-(1-34) partially inhibited the excitatory effect of Bay K-8644 on L channel currents. The final application of La^{+++} to the same cells blocked both T and L channel currents (Fig. VI-2), indicating that the pathway for Ba^{++} currents was Ca^{++} channels (Narahashi *et al.*, 1987). The concentration-related inhibition of L channel currents by bPTH-(1-34) is shown in Fig. VI-5. The threshold concentration of bPTH-(1-34) for inhibition of L channel currents was 0.1 μM, with 80% inhibition at 10 μM.

The effect of oxidized bPTH-(1-34) on L channel currents.

The oxidation of PTH leads to a loss of the biological effect of the hormone (Tashjian *et al.*, 1964). Likewise, the amino-terminal region of the intact PTH molecule, which contains all of the structural requirements for the known biological actions of PTH, is inactivated after oxidation, at least in terms of its action upon kidney (Rosenblatt *et al.*, 1977). Pang *et al.* (1983) showed that oxidized bPTH-(1-34) had no detectable vascular effect in rats and dogs. In the present investigation, oxidized bPTH-(1-34) had no effect on L channel currents in N1E-115 cells at a concentration of 1 µM. Untreated bPTH-(1-34) at the same final concentration of 1 µM was given immediately following the addition of H_2O_2-treated bPTH-(1-34). Under these conditions, untreated bPTH-(1-34) once again inhibited L channel currents in a distinctive manner. The absence of an inhibitory effect of H_2O_2-treated bPTH-(1-34) on L channel currents was confirmed in a total of 10 cells ($p>0.05$).

DISCUSSION

In order to ensure that the inhibitory effect of bPTH-(1-34) on the L channel currents was not due to some nonspecific effect of the peptide, an inactive form of bPTH-(1-34) was tested. In neuroblastoma cells, oxidized bPTH-(1-34) had no inhibitory action on the L channels. These channels could subsequently be inhibited by administration of untreated bPTH-(1-34) to the same cells. This provides strong evidence that the L channel-inhibiting effect of bPTH-(1-34) is an intrinsic property of this peptide fragment. The loss by oxidized bPTH-(1-34) of its inhibitory action on L channel currents is related, presumably, to the local charge changes introduced by oxidation of the methionines. The more negative charges imposed at the positions of methionines may sufficiently alter the molecules so that the part of the molecule responsible for the binding is not available to bind on the PTH receptors. In addition, PTH antagonists, such as bPTH-(3-34) and bPTH-(7-34), do not have a vascular effect or a calcium channel effect. This is shown in the following chapters. The difference between the effect of oxidized PTH preparation and PTH antagonists is that the former cannot bind to the PTH receptors while the latter can.

The results from patch clamp studies in N1E-115 cells were further supported by bPTH-(1-34) binding studies (Pang *et al.*,1990). The receptors for acetylcholine, histamine, 5-HT, dopamine, NGF, opiates and enkephalin, adenosine, and somatostatin have been identified in N1E-115 cells (Kimhi, 1981). The stimulation of the neurotensin receptor in N1E-115 cells activated inositol phospholipid metabolism and increased intracellular [Ca^{++}] (Snider *et al.*, 1986). Using a ^{125}I-labelled bPTH-(1-34) analog, 15-20% of the total binding of PTH analog by the membranes of the neuroblastoma cells was found to be specific. The specific binding was saturable.

The effect of bPTH-(1-34) on L channel currents in N1E-115 cells can also be correlated with changes in intracellular free calcium concentration determined by the fura-2 fluorescence technique (Pang *et al.*, 1990). In N1E-115 cells cultured in DMEM with 2% DMSO, a 15 mM KCl challenge increased intracellular free calcium concentration. It is possibly that this was mainly due to the opening of L channels. bPTH-(1-34) decreased this increase in intracellular Ca^{++}. In N1E-115 cells cultured in DMEM without the addition of DMSO, KCl also increased intracellular free calcium concentration which was presumably due to the opening of T channels. However, bPTH-(1-34) had no effect on the increase in intracellular free calcium concentration in this group of cells.

Unpublished observations from the laboratory of Dr. S. Harvey (Department of Physiology, University of Alberta) have suggested that PTH plays a role in the metabolism of dopamine in CNS. On the other hand, experiments on male rats indicated that centrally injected PTH inhibited the development of gastric ulcer (Clementi *et al.*, 1989). In addition, PTH administered i.c.v. also decreased gastric secretory volume and acid output. This further suggests the significant actions of PTH on certain areas of the CNS. The bPTH-(1-34)-elicited inhibition of L channel currents in N1E-115 cells, a spontaneous sympathetic tumour clone line, may be linked to the function of PTH in the neural system. Since L channels are believed to be a link between excitation and secretion, the PTH effect on L channels might provide a precise control over the release of some neurotransmitters or neuromodulators in the neural system.

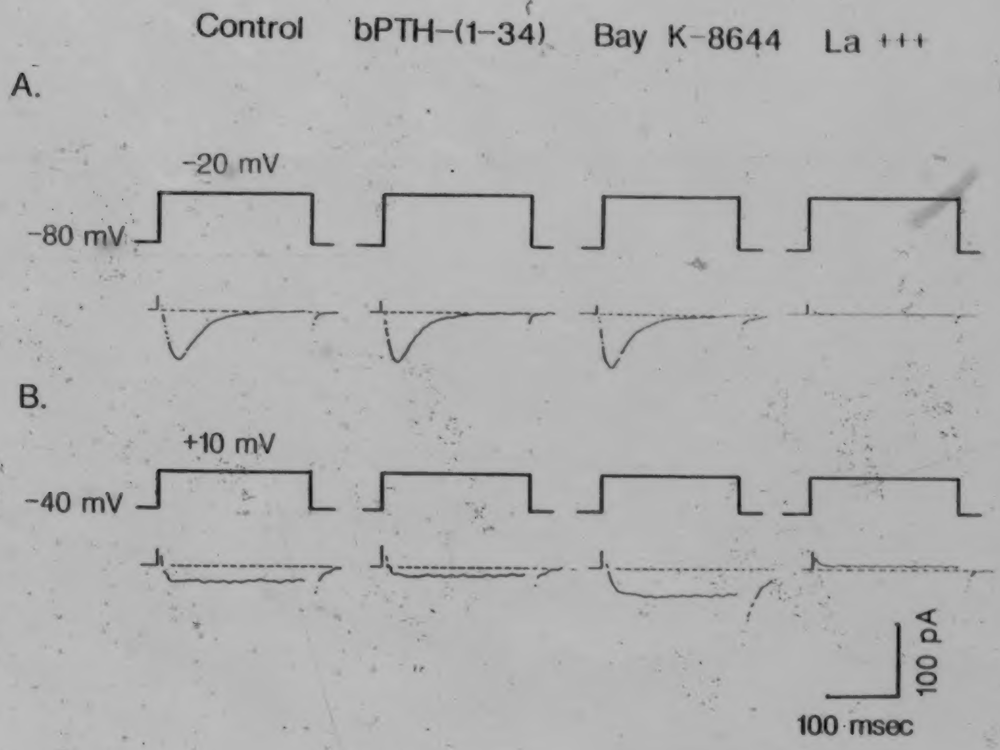

Control
bPTH-(1-34)
Bay K-8644
La +++
A.
-20 mV
-80 mV
B.
+10 mV
-40 mV
100 pA
100 msec

Fig. VI-1. Effects of bPTH-(1-34) and subsequently applied Bay K-8644 on T and L channel currents in N1E-115 cells. **A.** T channel currents had no response to 1 μM bPTH-(1-34) and sequentially applied 5 μM Bay K-8644. Cell #: 87521A. **B.** L channel currents were decreased in the presence of 1 μM bPTH-(1-34). This inhibitory effect of bPTH-(1-34) on L channel currents was antagonized by 5 μM Bay K-8644. The tail current was also enhanced by Bay K-8644. Cell #:87514E(2). La^{+++} at a concentration of 2 mM blocked both T and L channel currents as shown in A and B. Leakage and capacitive currents were not subtracted. Dashed lines indicate the zero level of currents.

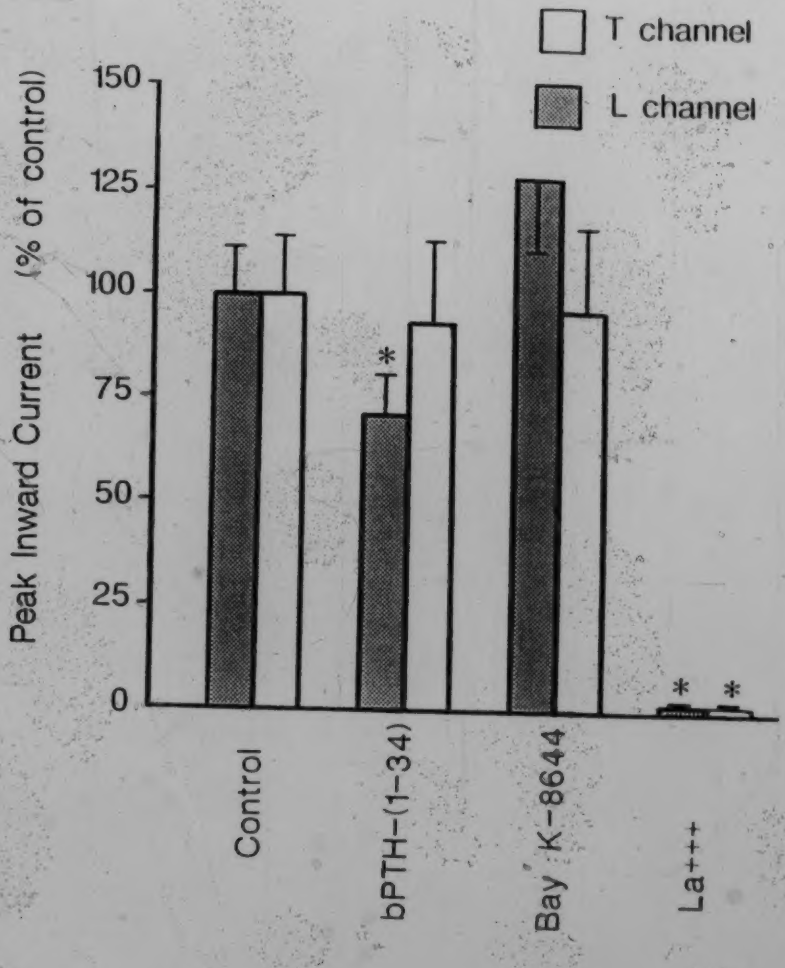

T channel
L channel
Peak Inward Current (% of control)
150
125
100
75
50
25
0
*
*
*
Control
bPTH-(1-34)
Bay K-8644
La+++

Fig. VI-2. The effect of bPTH-(1-34) on the peak amplitudes of T and L channel currents in N1E-115 cells. The experimental protocol was the same as that in Fig. VI-1. bPTH-(1-34) at a concentration of 1 μM had no effect on the amplitude of T channel currents ($n=8$) but decreased the amplitude of L channel currents ($n=9$). * indicates $p<0.05$ when compared with control or compared with the values after application of Bay K-8644.

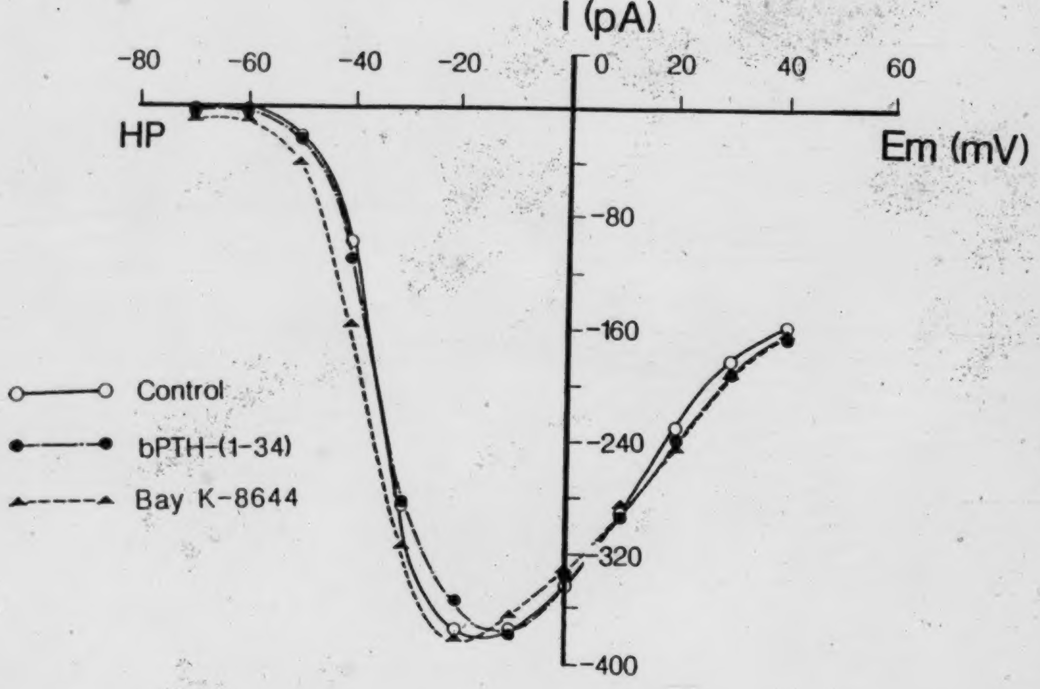

I (pA)
-80
-60
-40
-20
0
20
40
60
HP
Em (mV)
-80
-160
-240
-320
-400
Control
bPTH-(1-34)
Bay K-8644

Fig. VI-3. The effect of bPTH-(1-34) on the I-V relationship of T channel currents in one N1E-115 cell. After a stable control curve was obtained, 1 μM bPTH-(1-34) was added to the bath. Five minutes later, 5 μM Bay K-8644 was added to the same bath. Neither bPTH-(1-34) nor Bay K-8644 affected the amplitude or the I-V relationship of T channel currents. Leakage and capacitive currents have been subtracted during analysis.

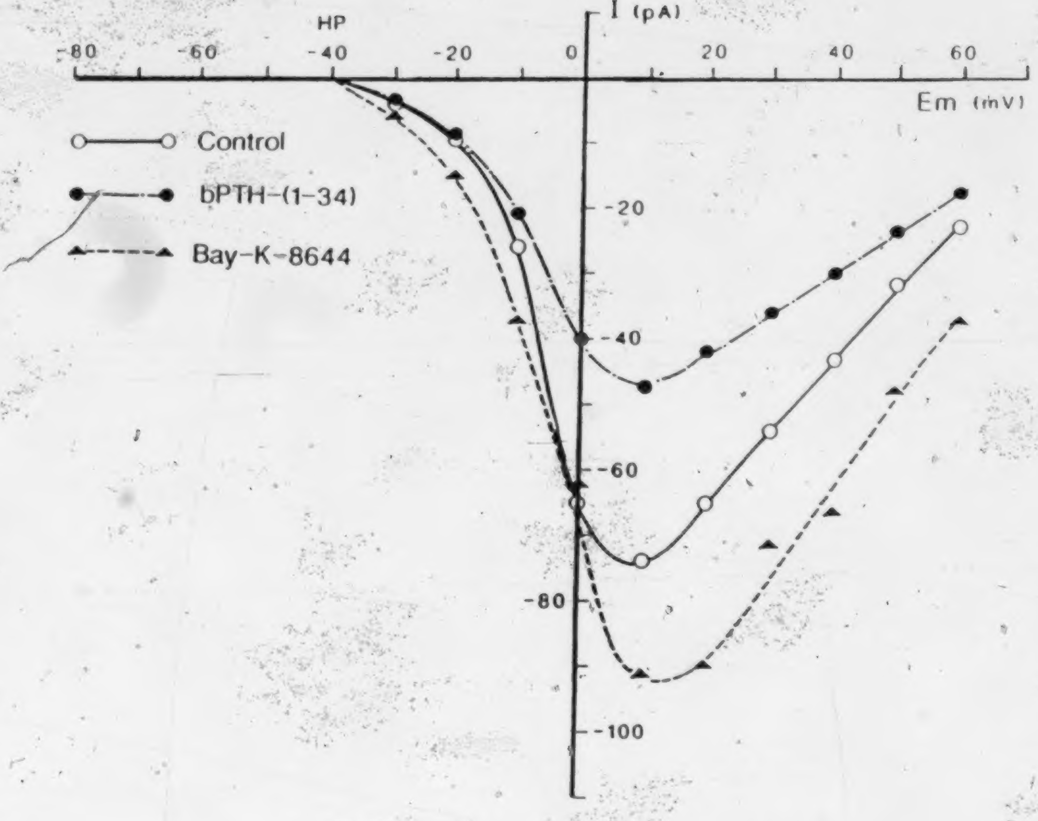

I (pA)
HP
-80
-60
-40
-20
0
20
40
60
Em (mV)
-20
-40
-60
-80
-100
Control
bPTH-(1-34)
Bay-K-8644

Fig. VI-4. The effect of bPTH-(1-34) on the I-V relationship of L channel currents in one N1E-115 cell. The experimental protocol was the same as described in Fig. VI-3. bPTH-(1-34) at 1 μM decreased the amplitude of L channel currents but did not affect the I-V relationship. Bay K-8644 at 5 μM increased the L channel currents but did not affect the I-V relationship, either. Leakage and capacitive currents have been subtracted during analysis.

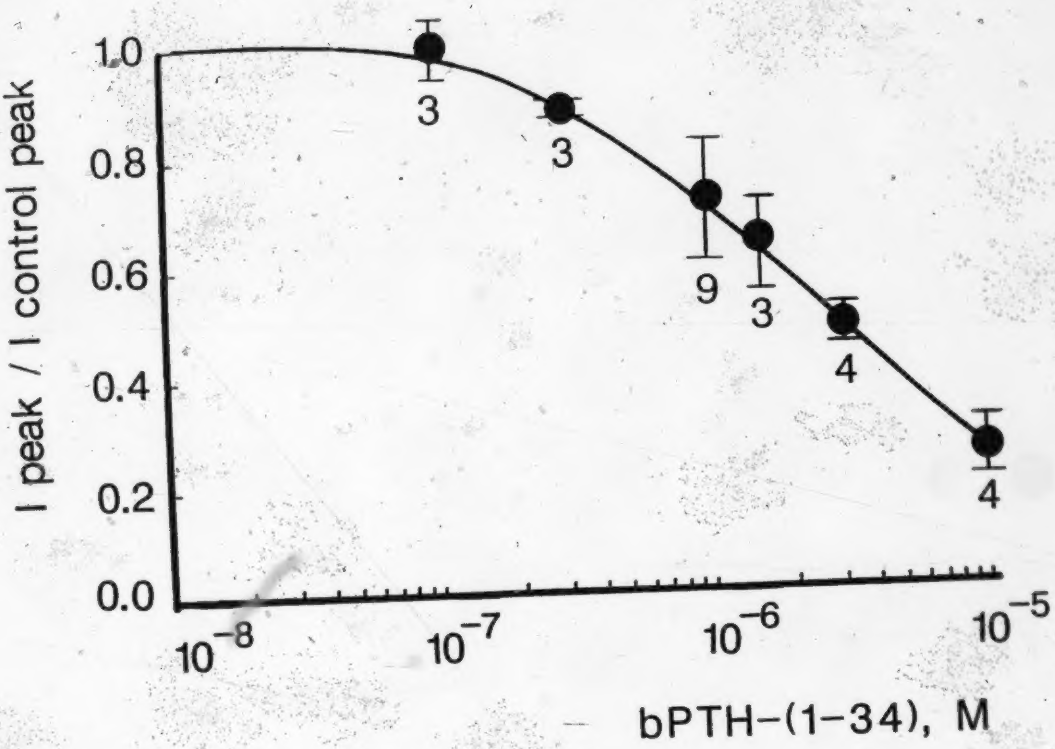

1.0
0.8
0.6
0.4
0.2
0.0
I peak / I control peak
3
3
9
3
4
4
10^{-8}
10^{-7}
10^{-6}
10^{-5}
bPTH-(1-34), M

Fig. VI-5. The concentration-related inhibition of L channel currents induced by bPTH-(1-34) in N1E-115 cells. The number of cells tested at each dosage is indicated on the concentration-response curve. The peak inward L channel current, activated from a holding potential of -40 mV, was measured in the absence and then presence of various concentrations of bPTH-(1-34). The current in the presence of bPTH-(1-34) (after 5 min) was compared as a ratio to the control value ($I_{peak}/I_{control\ peak}$). These normalized values were combined at each concentration and plotted as the mean ± SE.

CHAPTER 7

The selective inhibition of L channel currents by bPTH-(1-34) in rat tail artery smooth muscle cells

INTRODUCTION

Control of many calcium-dependent cellular events in excitable cells may depend on the modification of calcium influx via voltage-dependent calcium channels. Investigations using the patch clamp technique have identified two types of voltage-dependent calcium channel currents in rat tail artery smooth muscle cells (Wang *et al.*, 1989). The transient (T type) calcium channel current is activated at negative potentials and inactivates quickly and completely, while the long-lasting (L type) calcium channel current is activated at more positive membrane potentials and shows minimal inactivation. It has been suggested (Benham *et al.*, 1987) that the L channels represent the main population of voltage-dependent calcium channels in vascular smooth muscle cells and these channels may play a major role in the movement of Ca^{++} into the cell to trigger muscle contraction (Ohya and

Sperelakis, 1989).

Synthetic calcium antagonists have been used experimentally and clinically for more than a decade. Only recently, however, has the modulatory effect of endogenous hormones and peptides on calcium channels, which may be a physiologically important mechanism, received attention. Parathyroid hormone (PTH), for example, has been implicated in the modulation of voltage-dependent calcium channels in many cell types. As a potent vasodilator, bPTH-(1-34) inhibited KCl-stimulated vasoconstriction and $^{45}Ca^{++}$ uptake in rat tail artery strips (Pang *et al.*, 1984, 1988a).

In the present study, the effect of bPTH-(1-34) on the two types of calcium channel currents in rat tail artery smooth muscle cells are investigated. The specificity, reversibility, membrane potential dependence and the concentration dependence of the PTH effect are also examined. In addition, the effects of different fragments of PTH [bPTH-(3-34) and bPTH-(7-34)] and their interaction with bPTH-(1-34) are described.

RESULTS

The effect of bPTH-(1-34) on the T channel current

The experiment described in Fig. VII-1 shows that bPTH-(1-34) at a concentration of 1 μM had no effect on the peak amplitudes of T channel currents at all test potentials. This is shown in both the current records and the I-V relationship. In 5 cells with predominantly T channel currents, 1 μM bPTH-(1-34) failed to modulate T channel currents. The subsequent application of La^{+++} completely eliminated this transient inward current (Fig. VII-2).

The effect of bPTH-(1-34) on the L channel current

The magnitude of L channel currents was reduced in the presence of bPTH-(1-34). Usually, the decrease in the magnitude of L channel currents induced by bPTH-(1-34) was evident at 2-3 min and reached a steady state level within 5 min. In some cells ($n = 3$), after the initial 5 min decrease, the magnitude of L channel currents recovered to the control level, suggesting the existence of a mechanism of desensitization to bPTH-(1-34). Fig. VII-3 shows that, in one cell, bPTH-(1-34) (1 μM) inhibited the L channel current by 26.3%. The effect was more evident at the peak of the I-V relationship. In 14 cells, the maximum inward current was 74 ±13 pA and 49 ±10 pA before and after the application of 1 μM bPTH-(1-34), respectively ($p < 0.05$). The leakage current and the current required to hold the cell membrane at -40 mV were not changed by bPTH-(1-34).

To observe the effects of bPTH-(1-34) on the kinetics of L channel currents, the inward currents evoked from a holding potential of -40 mV in the absence or presence of bPTH-(1-34) were compared. When the reduced current records obtained in the presence of bPTH-(1-34) were scaled and superimposed on the inward current trace before application of bPTH-(1-34), identical traces were obtained (Fig. VII-3C), indicating that bPTH-(1-34) has no effect on the kinetics of L channel currents. In addition, the threshold potential for the activation of L channel currents, the potential at which the maximum inward currents occurred and the zero current potential (apparent reversal potential) for calcium channel currents were not changed in the presence of bPTH-(1-34). The unchanged reversal potential for L channel currents in the absence or presence of bPTH-(1-34) indicated that the reduction in L channel currents by bPTH-(1-34) was not due to changes in the intracellular concentration of free calcium.

The inhibition of L channel currents in the present study is mainly due to the specific effect of bPTH-(1-34) on L channel currents without the contamination of outward currents. The evidence for this is as follows: 1) the compositions of pipette solution (150 mM Cs^+) and bath solution (20 mM Ba^{++}) were designed to eliminate the voltage-dependent K^+ current and the Ca^{++}-dependent K^+ current (Quandt and Narahashi, 1984; Benham *et al.*,1985); 2) application of 2 mM La^{+++} revealed a linear relationship between voltage and current in the presence of bPTH-(1-34) over the range of voltages associated with K^+ current activation (Fig. VII-3A) (Dunlap and Fischbach, 1981); and 3) the steady-state activation curve was not shifted by bPTH-(1-34) (Fig. VII-9).

The inconsistent responsiveness of voltage-dependent calcium channels in many cases, such as the effect of calcitonin gene-related peptide on N-type (neuron) calcium channels (Ryu *et al.*, 1988), vasopressin on L channels (Mollard *et al.*, 1988), noradrenaline on L channels (Benham and Tsien, 1988) and isoproterenol on L channels (Droogmans *et al.*, 1987), has been reported. In rat tail artery smooth muscle cells, the responsiveness of L channels to bPTH-(1-34) was also not consistent. In a total of 46 cells, bPTH-(1-34) at different concentrations produced a decrease in L channel currents in 34 cells. Since only one cell was tested in one dish, one cannot be certain that all cells in the same dish had the same reactivity to bPTH-(1-34). Some of the many factors that may be responsible for the variability of the bPTH-(1-34) effect are considered in Chapter 11.

Interactions of bPTH-(1-34) and Bay K-8644

There are several reasons which justify the investigation of interactions of bPTH-(1-34) and Bay K-8644. First, Bay K-8644 is a specific L channel agonist, and thus can be used to examine the channel specific effect of other L channel modulators. Second, it has been reported that both the effects of Bay K-8644 (Tiaho *et al.*, 1990) and bPTH-(1-34) on L channel currents (see Chapter 10) are modulated by a cAMP-dependent mechanism. Third, Armstrong D. and Eckert (1987) demonstrated that Bay K-8644 modulated the gating of calcium channels only when the channel was phosphorylated. If the run-down of calcium channel currents was complete, the subsequent application of Bay K-8644 failed to modulate calcium channels. Hence, these authors suggested that the effect of Bay K-8644 on calcium channel currents depended on, and influenced the status, of phosphorylation-dephosphorylation of calcium channels. Fourth, the study of calcium channel modulation by β-adrenergic agents and Bay K-8644 has led to a critical understanding of the mechanisms of both agents (Tsien, *et al.*, 1986). Fifth, the similarity of the effects of Bay K-8644 and a peptide, isolated from spontaneous hypertensive rats, on voltage-dependent calcium channels has been reported (Huang *et al.*, 1988). These two agents were suggested to share a common mechanism of action although no fundamental data are available regarding the single channel characteristics in the presence of these two agents. Taken together, the elucidation of the possible interaction between the effects of bPTH-(1-34) and dihydropyridine agonists on L channels will help us to understand the mechanisms of channel modulation under physiological conditions and pharmacological intervention. In addition, the interaction of bPTH-(1-34) and Bay K-8644 on calcium channels in renal epithelial cells has been reported (Bacskai and Friedman, 1990).

Fig. VII-4 shows that Bay K-8644 increased the inward calcium channel currents which were previously inhibited by bPTH-(1-34). This result confirmed that the inward calcium channel currents affected by bPTH-(1-34) were, specifically, L channel currents. Further comparison of the amplification of L channel currents by Bay K-8644 in the presence or absence of bPTH-(1-34) was made. Without bPTH-(1-34), 5 μM Bay K-8644 enhanced the inward current to 252 ±37% of the control value. However, at the same concentration, Bay K-8644 increased the amplitude of L channel currents by 35 ±19% in the presence of 1 μM bPTH(1-34), compared to the current amplitude before the application of bPTH-(1-34) (Fig. VII-4).

Another experimental protocol is illustrated in Fig. VII-5. The experiment began with the first application of 1 μM bPTH-(1-34). Wash-out of the peptide followed. In this cell, the first application of bPTH-(1-34) decreased the inward current by 31% while the I-V relationship remained the same. Subsequent application of Bay K-8644 increased the inward current by a factor of 2.5. After the Bay K-8644 induced increase in L channel currents was maximal, 1 μM bPTH-(1-34) was again added to the bath solution. At this time, the inward current again decreased by 34%. Bay K-8644 shifted the peak of the I-V plot by 20 mV toward a more negative direction. This shift of the peak of the I-V curve was unchanged by the second application of bPTH-(1-34).

Furthermore, bPTH-(1-34) failed to modify the steady-state activation curve of L channels (Fig. VII-9) but Bay K-8644 shifted the curve toward the negative direction by 10 mV (Wang *et al.*, 1989). These results, as expected, suggest that bPTH-(1-34) affects L channels but in a different mechanisms from the actions of Bay K-8644.

Characteristics of the effect of bPTH-(1-34) on L channel currents

The effect of bPTH-(1-34) is concentration dependent. PTH lowered the blood pressure *in vivo* and inhibited tension development in strips from rat tail artery *in vitro* in a concentration-dependent manner (Pang *et al.*, 1988a). Whether the effect of PTH on the inward current in single smooth muscle cells could be related to the concentration used was examined in the present study. Fig. VII-6 illustrates a concentration-dependent inhibition of L channel currents by bPTH-(1-34). At a concentration of 0.1 μM, bPTH-(1-34) decreased the peak inward currents to 81.1 ± 3% of the control values in 7 cells ($p < 0.05$). The threshold concentration for inhibitory action of bPTH-(1-34) is, therefore, < 0.1 μM. When the concentration of bPTH-(1-34) was raised to 10 μM, 59.6 ± 6% of the L channel current still remained.

The effect of bPTH-(1-34) is voltage dependent. It has been reported that, by changing the membrane potential, the inhibitory effect of some calcium antagonists on L channel currents could be modified, such as in smooth muscle cells from rat mesenteric arteries (Bean *et al.*, 1986) and neuroblastoma cells (Ogata *et al.*, 1989). Hence, experiments were designed to determine the voltage dependence of the bPTH-(1-34) effect on L channel currents in rat tail artery smooth muscle cells.

At first, the effect of bPTH-(1-34) at two different holding potentials, -60 and -20 mV, was investigated. Fig. VII-7 shows the results from one smooth muscle cell. When the holding potential was set at -20 mV, the peak inward current was decreased by 34.5% by bPTH-(1-34) at a concentration of 1 μM. However, with the holding potential at -60 mV, the peak inward current was not depressed in the presence of bPTH-(1-34). This result suggested that bPTH-(1-34) inhibited L channel currents more strongly at more positive holding potentials. Similar findings were observed in 3 other cells.

This mechanism was examined in more detail by generating the full range of steady-state inactivation curves in the absence or presence of bPTH-(1-34). The holding potential was changed from -80 to +10 mV and the step command was applied to depolarize the membrane to +20 mV. The membrane was held for 15 seconds at each holding potential set. The steady-state inactivation curves were plotted using either the absolute value of the peak inward current as in Fig. VII-8A or the normalized amplitude of the inward currents as in Fig. VII-8B against the holding potentials. The Boltzmann distribution of the form: $I = I_{max} [1 + \exp (V - V_h)/k]^{-1}$ was used to fit the steady-state inactivation curves in Fig. VII-8B. In this equation, V represents the holding potential, V_h is the potential at which one-half of the calcium channels are inactivated and k is the slope factor. bPTH-(1-34) at a concentration of 1 μM decreased the amplitude of L channel currents at all the holding potentials tested (Fig. VII-8A) but its effect was more potent at more positive holding potentials (Fig. VII-8B). When the holding potential was within the range of -30 to -10 mV, the inhibition of L channel currents as well as the negative shift of the steady-state inactivation curve in the presence of bPTH-(1-34).

were more evident. It can be seen from Fig. VII-8A and 8B that bPTH-(1-34) shifted the steady-state inactivation curve to the left. The same phenomenon occurred in the presence of dihydropyridines, such as Bay K-8644 (Wang *et al.*, 1989) and nicardipine (Terada *et al.*, 1987b). The slopes of the relative inactivation curves (least square fit to the experimental data) shown in Fig. VII-8B are 8.7 and 11.5 mV in the absence or presence of bPTH-(1-34), respectively. V_h changed from -14.9 mV to -24 mV before and after the application of the peptide, respectively.

To examine further the voltage dependence of L channel current activation, the normalized conductance curves in the absence or presence of bPTH-(1-34) were compared (Hodgkin and Huxley, 1952b). These curves were plotted as relative conductance of L channels against the test pulses, and fitted by the Boltzmann distribution of the form: $G = G_{max} [1 + \exp(V_h - V) / k]^{-1}$, where V is the test potential which drives the inward current, V_h is the potential at which the half maximum conductance was obtained and k is the slope of the fitted curve. The normalized conductance of L channels in this cell preparation was a steep function of voltage, and increased markedly over the potential range from 0 to 20 mV. Fig. VII-9 shows that, in one cell, the conductances of L channels as a function of potential in the absence or presence of bPTH-(1-34) were identical. In another 5 cells examined, bPTH-(1-34) also produced no change either in the V_h values or the slope factors of the normalized conductance curves of L channels.

The effect of bPTH-(1-34) is not dependent on the pulse lengths. All the experiments described above were executed with a 200 msec depolarization pulse. It may be argued that bPTH-(1-34) could bind more tightly to L channels if the channel was the binding site of PTH and the channel remained in the activated state for a longer period of time. To clarify this point, the depolarizing pulse was prolonged to 3 or 10 seconds. In the absence of bPTH-(1-34), there were no differences among the amplitudes of L channel currents with 200 msec, 3 sec or 10 sec pulse lengths. After application of bPTH-(1-34), the amplitude of L channel currents decreased to the same extent with different pulse lengths. In addition, the duration of the interpulse interval, which varied from 20 msec to 5 sec, also did not influence the effect of bPTH-(1-34) on the amplitude of L channel currents.

The effect of bPTH-(1-34) on L channel currents is reversible. After the inhibition of L channel currents by bPTH-(1-34) was established, the cell was then perfused with PTH-free bath solution to check the reversibility of the bPTH-(1-34) effect. A satisfactory washout of bPTH-(1-34) was obtained with a perfusion rate of approximately 1 ml/10 sec. As shown in Fig. VII-10, the peak amplitude of the L channel currents recovered to 93% of the control value after a total 5 min of washout and recovery.

The effect of bPTH-(1-34) on the deactivation of L channel currents. Immediately following the end of a depolarization pulse, the calcium channel deactivated in response to repolarization to generate an instantaneous current or "tail current". The amplitude of tail currents reflects the instantaneous activity of the channel and the time course represents the kinetics of the closing process(es) of calcium channels. In the present study, the tail currents mainly measured the deactivation of L-type calcium channels because a holding potential of -40 mV inactivated T-type calcium channels and a 40 msec pulse fully activated L channels but inactivated most T channels. When the membrane was repolarized ~~to~~ -40 mV from different test pulses, the time course of the tail current decay was well fitted by a single exponential function (Fig. VII-11A), which is comparable to the tail current of L channels in neuroblastoma cells (Yoshii *et al.*, 1988).

In view of this, I measured the instantaneous amplitude of the tail current at time zero, the instant at which the repolarization was initiated, by extrapolating the falling phase of the tail current with a single exponential function. The current-voltage relationships of the tail currents were constructed and are shown in Fig. VII-11B (square symbols).

Quite different from the bell shape of the I-V relationship obtained during the test pulse, the sigmoidal I-V plot could be determined for the instantaneous amplitude of the tail current when repolarized to the holding potential from the step depolarizations. The amplitude of the tail current approached a plateau at +80 mV (Eckert and Douglas, 1983).

The change in the amplitude of the instantaneous tail current is proportional to that of the instantaneous calcium channel conductance. As expected, application of bPTH-(1-34) decreased the amplitude of the instantaneous tail current to the same degree as the decrease in the amplitude of the peak inward current during the test pulse (Fig. VII-11A) in the presence of the peptide. However, the decay of the tail current upon repolarization was not affected by bPTH-(1-34) (Fig. VII-11 A-a), suggesting that the gating mechanism controlling the closing process of L channels was not altered by the peptide. The time constants of tail currents after being depolarized to +30 mV were 5.22±0.5 msec in the absence, and 5.60±0.4 msec in the presence, of bPTH-(1-34) (n=5).

Effects of bPTH-(3-34) and bPTH-(7-34) on L channel currents

In an attempt to compare the effect of different fragments of bPTH on L channel currents, bPTH-(3-34) and bPTH-(7-34) were used in the next group of experiments. When the time course of changes in the magnitude of L channel currents in response to bPTH-(3-34) and bPTH-(7-34) was followed, up to 5 min after the addition of these two fragments, neither a decrease nor increase in the amplitude of L channel currents was seen. bPTH-(3-34) at a concentration of 1 μM had no effect during the 5 min period of application. I-V curves and original current traces are shown in Fig. VII-12. Fig. VII-14 shows that 10 μM bPTH-(7-34) in 7 cells or 1 μM bPTH-(3-34) in 15 cells did not affect L channel currents. No change in the rate of activation or inactivation of L channel currents could be detected in the presence of bPTH-(3-34) or bPTH-(7-34).

The effect of pretreatment of cells with bPTH-(3-34) on subsequent changes in L channel currents induced by bPTH-(1-34)

To examine whether the change in L channel currents induced by bPTH-(1-34) is exerted via a PTH specific receptor(s), the effect of bPTH-(1-34) on the L channel current after pretreatment of cells with bPTH-(3-34), a PTH antagonist, is tested. It was shown that bPTH-(3-34) did not modify L channel currents by itself (Fig. VII-12). However, when one cell, shown in Fig. VII-13, was pretreated with 1 μM bPTH-(3-34) for more than 10 min, the addition of bPTH-(1-34) (1 μM) was ineffective in terms of the inhibition of L channel currents. The data from this cell show that bPTH-(3-34) is capable of blocking the inhibitory effect of bPTH-(1-34) on L channels. Results from more cells further supported this hypothesis (Fig. VII-14). In 10 cells, the L channel current had a magnitude of 40.3 ± 5.0 pA after pretreatment of the cells with 1 μM bPTH-(3-34). Five min after the addition of 1 μM bPTH-(1-34) to the bath solution, the magnitude of L channel currents was 36.2 ± 7 pA. There was no significant difference in channel currents between these two treatments.

The absence of an inhibitory effect on L channel currents in smooth muscle cells by bPTH-(1-34) may be explained if bPTH-(3-34) occupies the same binding sites as bPTH-(1-34) and thus decreased bPTH-(1-34) binding. It is also possible that bPTH-(3-34) binding changed the configuration of the L channel complex in such a way that the L channels lost their pharmacological response to the subsequent application of any agent. If the latter were the case, the subsequent challenge to the L channel by Bay K-8644 would be ineffective. However, the results shown in Fig. VII-12 indicated that Bay K-8644 was still able to enhance L channel currents, suggesting a normal responsiveness of L channel proteins and the existence of different binding sites on or near L channel proteins for PTH fragments and Bay K-8644.

DISCUSSION

Significance of the present study

Since the introduction of calcium antagonists and agonists more than a decade ago, a variety of studies have been conducted to determine the effects of these exogenous compounds on voltage-dependent calcium channels. From these studies, the pharmacological criteria for identification of different types of voltage-dependent calcium channels have been established (Bean, 1989a, b), and the biochemical purification of calcium channel protein became feasible (Hofmann *et al.*, 1988). In addition, these agents have been employed in the clinical treatment of some cardiovascular and neural disorders (Olivari *et al.*, 1979; Greenberg, 1987). However, the mechanisms by which living organisms regulate the activity of voltage-dependent calcium channels are still not clear. It is reasonable to postulate the existence of endogenous calcium channel modulators, both antagonists and agonists, which modulate calcium channels under physiological and pathophysiological conditions. Endothelin, a peptide produced by vascular endothelial cells, has been demonstrated to activate L-type voltage-dependent calcium channels in vascular smooth muscle cells, either directly or indirectly (Goto *et al.*, 1989). In the present study, it is reported that the active N-terminal fragment of PTH, bPTH-(1-34), inhibits L channel currents in single smooth muscle cells from the rat tail artery. The inhibitory effect of bPTH-(1-34) is concentration-dependent and voltage-dependent, but does not rely on the frequency and duration of depolarization, at least within the range tested in the present study. PTH, then, is the first endogenous circulating peptide hormone found capable of inhibiting (modulating) voltage-dependent calcium channels in vascular smooth muscle cells. This finding will lead to a more critical evaluation of the mechanism of PTH action. The methodology and protocols established in this study will also assist in the identification of other endogenous substances responsible for the regulation of voltage-dependent calcium channels.

Correlation with in vivo and in vitro studies

As previously reported, PTH lowers blood pressure *in vivo*. The tension development in rat tail artery strips, *in vitro*, elicited by 60 mM KCl is attenuated by PTH. This PTH-induced effect was reduced by the removal of extracellular

Ca^{++} and mimicked by calcium channel blockers, such as D-600. The identification of two types of voltage-dependent calcium channels in rat tail artery cells opens the possibility that PTH exerts its vasoactive action, at least in part, by modulating the influx of calcium through these channels. The present results provide direct evidence to support the view that PTH inhibits calcium channels in vascular smooth muscle cells. This was previously suggested by a tension and calcium flux study at the tissue level (Pang *et al.*, 1988a). The rat tail artery possesses contractile properties similar to those of small resistance vessels (Frost *et al.*, 1976). The activation of L type calcium channels in the smooth muscle cells from rat tail artery might be the key link between excitation and contraction of this cell preparation. Consequently, the inhibition of L channels by bPTH-(1-34) blocks the access of extracellular calcium to the intracellular space of the cell. I believe this is part of the mechanism of PTH-induced vasodilatation (Pang *et al.*, 1988a, 1990). Since PTH has different roles in various target organs and calcium channels do not behave in the same way in all tissues (Bean *et al.*, 1986), it is reasonable to suggest that PTH may regulate calcium channels in a somewhat different manner in other tissues. In addition, the presence of PTH receptors on vascular smooth muscle cells has been demonstrated (Nickols *et al.*, 1989b, 1990).

Mechanisms of the inhibitory effect of bPTH-(1-34) on L channel currents

Although the mediation of L channel currents by bPTH-(1-34) is significant, it is still not known whether the suppression of L channel currents is due to the direct action of bPTH-(1-34), *per se*, on calcium channel protein or to the activation of some cytoplasmic second messengers through the binding of PTH to its specific receptor(s) outside the calcium channel protein(s). It is not possible to distinguish between these two possibilities from the data presented in this chapter. Furthermore, it remains possible that, under *in vivo* conditions, PTH-induced modulation of calcium channel currents is secondary to PTH-induced changes in the membrane potential, which occur independently of changes in Ca^{++} permeability (Koch *et al.*, 1988). It should also be pointed out that PTH might interact with some transducer macromolecules such as membrane GTP-binding proteins (G proteins) and inhibit L channel currents indirectly. Unfortunately, little is known regarding the role of G proteins in the modulation of voltage-dependent calcium channels in vascular smooth muscle cells (Zeng *et al.*, 1989).

The phosphorylation-dephosphorylation mechanisms in the regulation of voltage-dependent calcium channels have received increasing attention. The bPTH-(1-34)-induced inhibition of L channel currents may be mediated by cyclic AMP, since cyclic AMP is a potent relaxant for many smooth muscles (Hardman, 1981). In cultured vascular smooth muscle cells, 1 min treatment with PTH resulted in 5- to 10-fold increases in cyclic AMP concentrations (Nickols, 1985). It has been reported that the hypotensive action of bPTH-(1-34) may involve cellular cyclic AMP as a second messenger (Helwig *et al.*, 1984). bPTH-(1-34) increased cyclic

AMP content significantly in rat tail artery tissues and the vasodilatatory action of bPTH-(1-34) was potentiated by isobutylmethylxanthine, a phosphodiesterase inhibitor, and decreased by imidazole, a phosphodiesterase stimulator (Pang *et al.*, 1986). Detailed studies and discussions which correlate the effect of PTH on voltage-dependent calcium channels with the intracellular cyclic AMP level are described in Chapter 10.

The antagonistic action of bPTH-(3-34) to bPTH-(1-34)

A synthetic bovine PTH fragment containing the first 34 amino acid residues of the amino-terminus, bPTH-(1-34), has full biological activity in classical bioassays of PTH (see reviews by Rosenblatt, 1986 and Mok *et al.*, 1989). Some synthetic fragments of PTH shortened at the N terminus by only a few amino acids, such as bPTH-(3-34) and bPTH-(7-34), lost PTH agonist activity especially *in vitro* (Rosenblatt *et al.*, 1977). In renal epithelial cells, bPTH-(1-34) activated calcium channels. However, bPTH-(3-34) had no effect (Bacskai and Friedman, 1990). In many cell preparations, bPTH-(3-34) has been demonstrated to be a competitive antagonist of PTH (see review by Rosenblatt, 1986; Segre *et al.*, 1985; Civitelli *et al.*, 1989; Mine *et al.*, 1989; Tamura *et al.*, 1989; Bacskai and Friedman, 1990). This antagonist inhibits PTH-induced cAMP and adenylate cyclase responses, as well as vasorelaxation (Huang and Rorstad, 1984; Nickols, 1985). Although bPTH-(3-34) lacks hypotensive activity (Ellison and McCarron, 1984), it may still bind to PTH receptors in vascular smooth muscle preparations (Mok *et al.*, 1989a). *In vivo*, the dilator response of second-order arterioles to bPTH-(1-34) was abolished by bPTH-(3-34) (Dowe and Joshua, 1987). *In vitro*, the adenylate cyclase response of renal microvessels to bPTH-(1-34) was also inhibited by bPTH-(3-34) (Helwig, *et al.*, 1987). The inhibition by bPTH-(3-34) and bPTH-(7-34) of PTH-induced cAMP and adenylate cyclase responses, as well as vasorelaxation have also been reported (Huang and Rorstad 1984; Nickols, 1985; Daugirdas *et al.*, 1987).

PTH is a charged peptide and is, therefore, not lipid soluble. It might bind only to specific receptors on the external side of the membrane and then modify the calcium channel. This hypothesis can be demonstrated, at least in part, by the antagonism of the effect of bPTH-(1-34) by bPTH-(3-34) which, by itself, had no effect on L channel currents. Whether bPTH-(3-34) and bPTH-(1-34) have the same type of receptors in vascular smooth muscle cells is not clear at the present time. However, it has been shown that, in bovine middle cerebral arteries, an equal molar concentration of bPTH-(3-34) could block approximately half the vasorelaxant effect of bPTH-(1-34) (Suzuki *et al.*, 1983). Nickols *et al.* (1987) reported that a 50 times greater concentration of bPTH-(3-34) was required to abolish bPTH-(1-34)-induced relaxation in rabbit aortic strips. In the present study, the same concentration of bPTH-(3-34) and bPTH-(1-34), 1 μM, was used. A concentration of 1 μM of bPTH-(3-34) was fully effective in producing complete inhibition of 1 μM of bPTH-(1-34). This phenomenon can be explained if the binding sites for bPTH-(3-34) on the membrane or the secondary events induced by bPTH-(3-34) inside the membrane are saturated at a concentration of 1 μM for bPTH-(3-34). Alternatively, bPTH-(3-34) at the same concentration as bPTH-(1-34) may not completely block the binding of bPTH-(1-34), but can completely abolish the effect of bPTH-(1-34) on voltage- and time-dependent calcium channel currents in a single cell. However, the tension development in the experimental arterial strips was induced by $PGE_{2\alpha}$ (Suzuki *et al.*, 1983) or norepinephrine (Nickols *et al.*, 1987). It would be more reasonable to suggest that the tension development in these reports was triggered by the intracellular release of free Ca^{++} or the activation of receptor-operated calcium channels as well as the opening of voltage-dependent calcium channels. It is also important to recognize that the contraction of vascular smooth muscle is controlled by multiple factors: not only extracellular calcium influx but also intracellular calcium release (see review by Van Breemen and Saida, 1989), and phosphorylation of the myosin light chain by a Ca^{++}/calmodulin-dependent myosin light chain kinase (see review by Kamm and Stull, 1989). Given different mechanisms underlying excitation-contraction coupling in smooth muscles, the complete inhibition of the effect of bPTH-(1-34) on L-type voltage-dependent calcium channel currents by bPTH-(3-34) at an equal molar concentration would be acceptable.

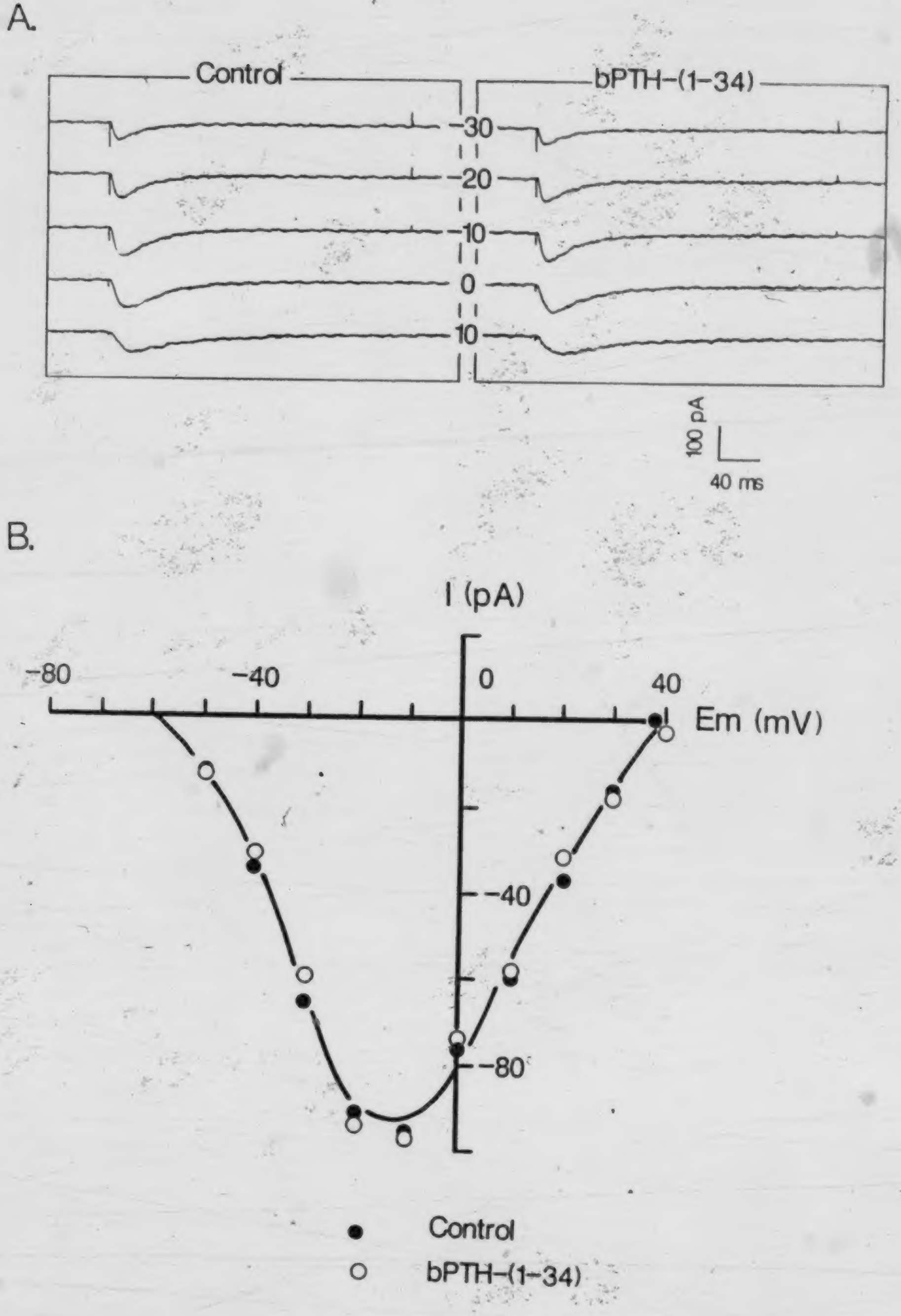

A.
Control
bPTH–(1–34)
−30
−20
−10
0
10
100 pA
40 ms
B.
I (pA)
−80
−40
0
40
Em (mV)
−40
−80
● Control
○ bPTH–(1–34)

Fig. VII-1 The effect of bPTH-(1-34) on T channel currents in one vascular smooth muscle cell. **A.** The original current traces were elicited from a holding potential of -80 mV to various test pulses indicated beside each trace (mV). **B.** I-V relationships of T channel currents were constructed before (open circles) and after (solid circles) the addition of 1 μM bPTH-(1-34), respectively. The same cell was used in **A** and **B**. Leakage and capacitive currents have been corrected.

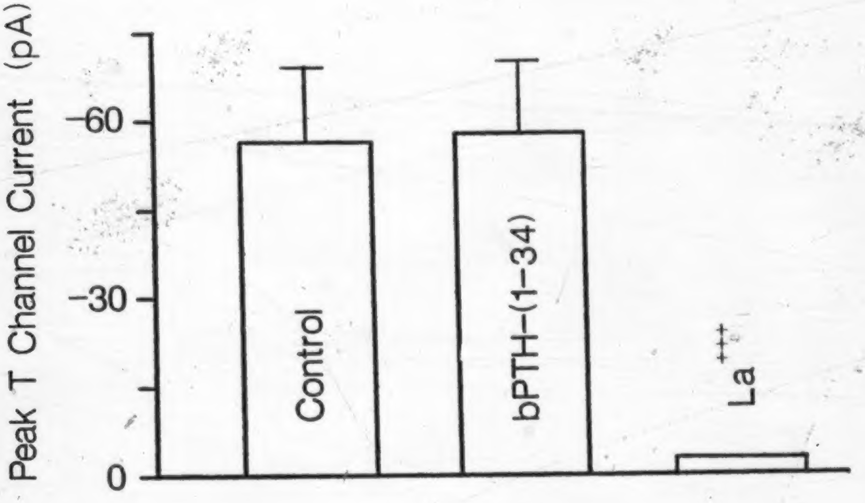

Peak T Channel Current (pA)
-60
-30
0
Control
bPTH-(1-34)
La+++

Fig. VII-2 T channel currents in vascular smooth muscle cells were not sensitive to bPTH-(1-34). Data from 5 cells were combined and shown as a bar graph. bPTH-(1-34) at a concentration of 1 μM did not change the peak amplitudes of T channel currents ($p > 0.05$). 2 mM La^{+++} eliminated T channel currents.

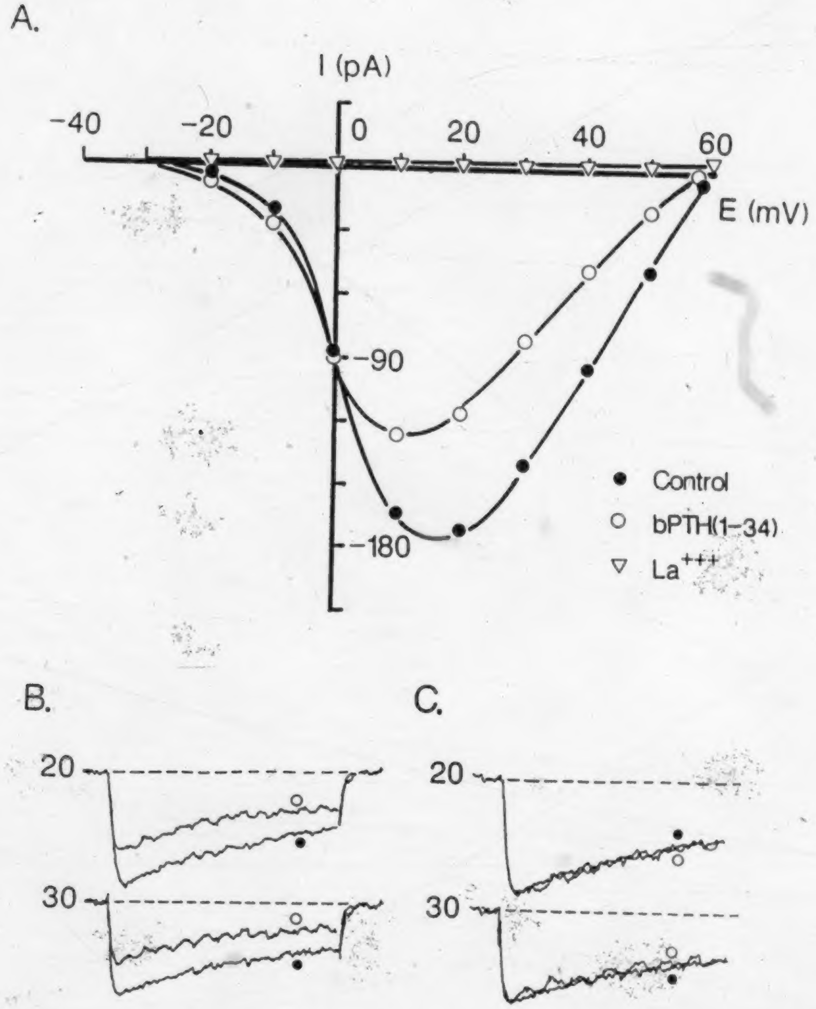

A.
I (pA)
−40
−20
0
20
40
60
E (mV)
−90
−180
● Control
○ bPTH(1−34)
▽ La+++
B.
20
30
50 pA
50 mesc
C.
20
30
50 pA
50 pA
50 msec

Fig. VII-3 The inhibitory effect of bPTH-(1-34) on L channel currents in one smooth muscle cell from the rat tail artery. **A.** The I-V relationships under different experimental conditions. After stable currents were obtained as a control (solid circles), 1 μM bPTH-(1-34) was added to the bath for 5 min (open circles) and followed by 2 mM La^{+++} (open triangles). The application of La^{+++} completely blocked the inward current, which is shown by the linear I-V relationship. **B.** The original current traces before and after the addition of bPTH-(1-34). **C.** The current traces in the presence of bPTH-(1-34) were re-scaled and superimposed with the control current traces with the same scale as in **B**. The superimposed currents showed that bPTH-(1-34) did not change the kinetics of inactivation of L channel currents. Leakage and capacitative currents have been subtracted. Holding potential: -40 mV.

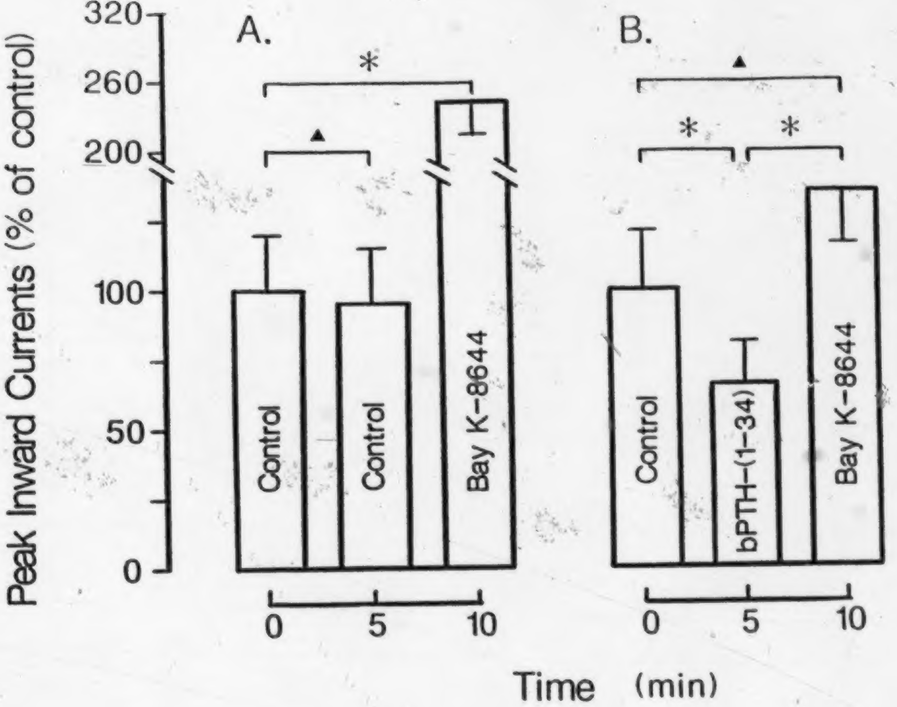

A.
B.
Peak Inward Currents (% of control)
320
260
200
100
50
0
Control
Control
Bay K-8644
Control
bPTH-(1-34)
Bay K-8644
*
▲
*
*
▲
0
5
10
0
5
10
Time (min)

Fig. VII-4 The amplification of L channel currents by Bay K-8644 in the absence or presence of bPTH-(1-34) in vascular smooth muscle cells. **A.** The control value was measured first. Five min later the peak inward current was measured again, and then 5 μM Bay K-8644 was applied. At the 10th min the current amplitude was recorded (n = 7). **B.** The peak inward current was recorded as control and then 1 μM bPTH-(1-34) was added. At 5 min, the peak inward current in the presence of bPTH-(1-34) was measured and 5 μM Bay K-8644 was added. The Bay K-8644-induced change in the current amplitude was recorded at the 10th min (n = 10). * $p<0.05$. ▲ $p>0.05$.

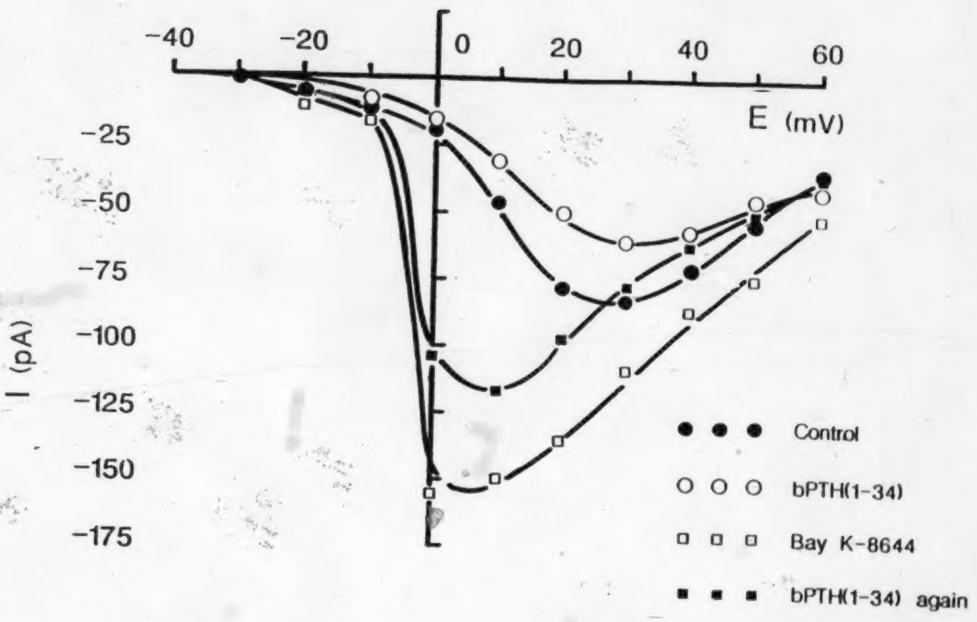

-40
-20
0
20
40
60
E (mV)
-25
-50
-75
-100
-125
-150
-175
I (pA)
● ● ● Control
○ ○ ○ bPTH(1-34)
□ □ □ Bay K-8644
■ ■ ■ bPTH(1-34) again

Fig. VII-5 The interaction of bPTH-(1-34) with Bay K-8644 in vascular smooth muscle cells. The solid circles represent the I-V relationship of L channel currents obtained before the addition of bPTH-(1-34). The first addition of 1 μM bPTH-(1-34) (open circles) decreased L channel currents by 31%. After the peptide was washed out of the bath, subsequent application of 5 μM Bay K-8644 (open squares) increased the L channel current. The second addition of 1 μM bPTH-(1-34) (solid squares) once again depressed L channel currents that had been increased by Bay K-8644. Holding potential: -40 mV.

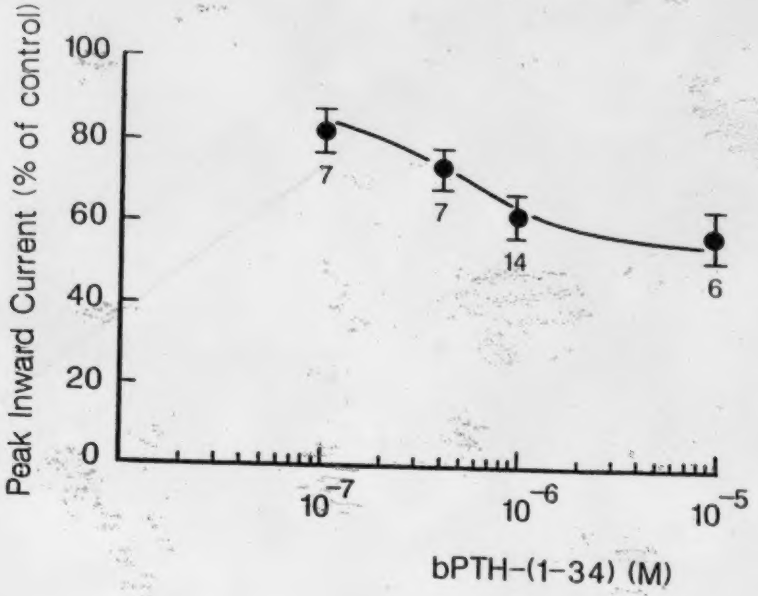

Peak Inward Current (% of control)
100
80
60
40
20
0
7
7
14
6
10^-7
10^-6
10^-5
bPTH-(1-34) (M)

Fig. VII-6 Concentration response of L channel currents in vascular smooth muscle cells to bPTH-(1-34). The number of cells tested at each concentration is indicated by each point on the concentration-response curve. bPTH-(1-34) significantly reduced the inward currents at all concentrations tested ($p < 0.05$). Statistical significance was determined by analysis of variance in conjunction with the Newman-Keul's test. The difference is also significant among the 0.1, 0.3 and 1 μM groups but not between the 1 and 10 μM groups.

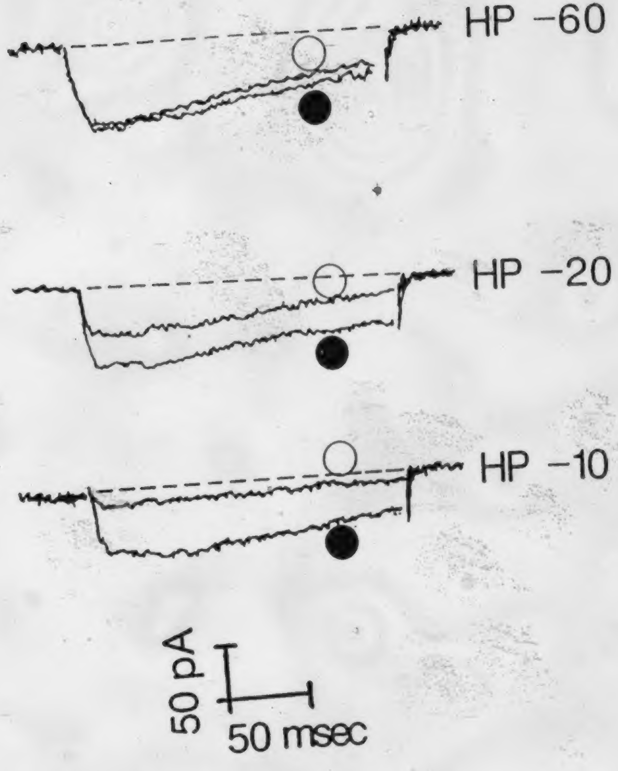

HP -60
○
●
HP -20
○
●
HP -10
○
●
50 pA
50 msec

Fig. VII-7 The effect of bPTH-(1-34) on L channel currents elicited from various holding potentials (mV) in vascular smooth muscle cells. Currents were elicited with a test potential to +30 mV before (filled circles) and after (open circles) the addition of bPTH-(1-34) in one cell. Leakage and capacitative currents have been subtracted.

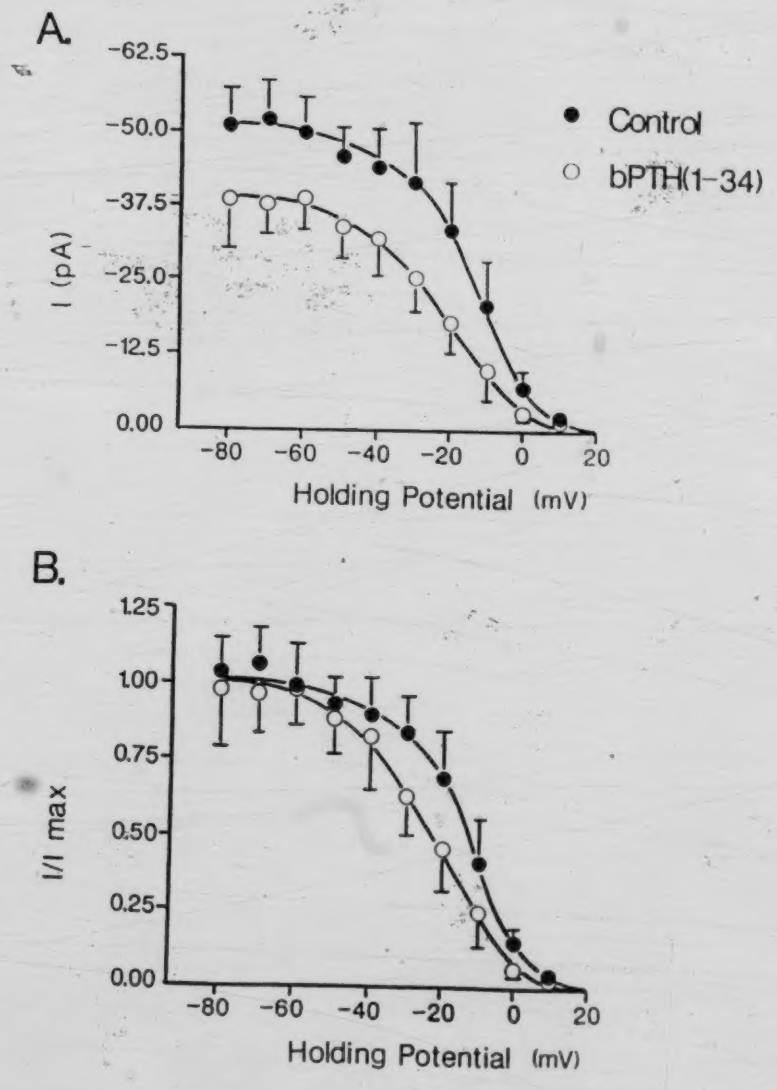

A.
-62.5
-50.0
-37.5
-25.0
-12.5
0.00
I (pA)
-80
-60
-40
-20
0
20
Holding Potential (mV)
● Control
○ bPTH(1-34)
B.
1.25
1.00
0.75
0.50
0.25
0.00
I/I max
-80
-60
-40
-20
0
20
Holding Potential (mV)

Fig. VII-8. The steady-state inactivation curves of L channel currents in vascular smooth muscle cells before (solid circles) and after (open circles) the addition of 1 μM bPTH-(1-34). **A.** The steady-state inactivation of L channels plotted by using absolute amplitude of inward currents. The data from six cells was combined and plotted as the X ±SE (n = 6). The data shown in **A** were replotted in **B** using the normalized amplitude of inward currents. The inactivation curves in **B** were fitted by the least squares method to the Boltzmann equation: $I=I_{max}[1+\exp(V-V_h)/k]^{-1}$. Before the addition of bPTH-(1-34), V_h=-14.9 mV and k=8.7 mV. After the addition of the peptide, V_h=-24.0 mV and k=11.5 mV. At holding potentials from -30 to -10 mV the differences between the two curves are significant ($p<0.05$).

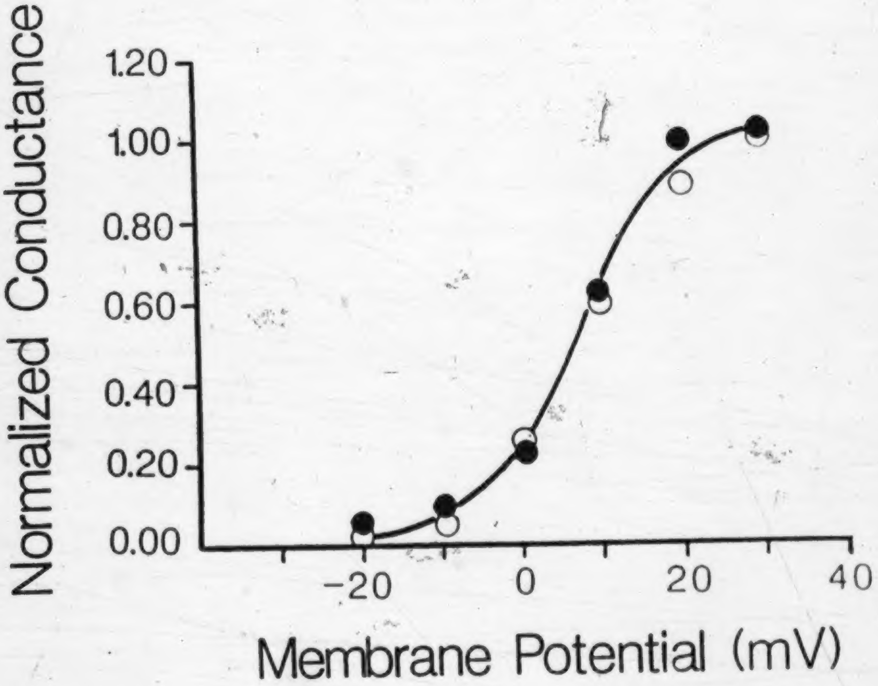

Normalized Conductance
1.20
1.00
0.80
0.60
0.40
0.20
0.00
-20
0
20
40
Membrane Potential (mV)

Fig. VII-9. The normalized conductance curves of L channels in the absence (filled circles) or presence (open circles) of 1 μM bPTH-(1-34) in one vascular smooth muscle cell. The least squares fit to the control data by the Boltzmann distribution of the form: $G = G_{max}[1+\exp(V_h-V)/k]^{-1}$ is the solid curve, with V_h=7.8 mV and k=6 mV.

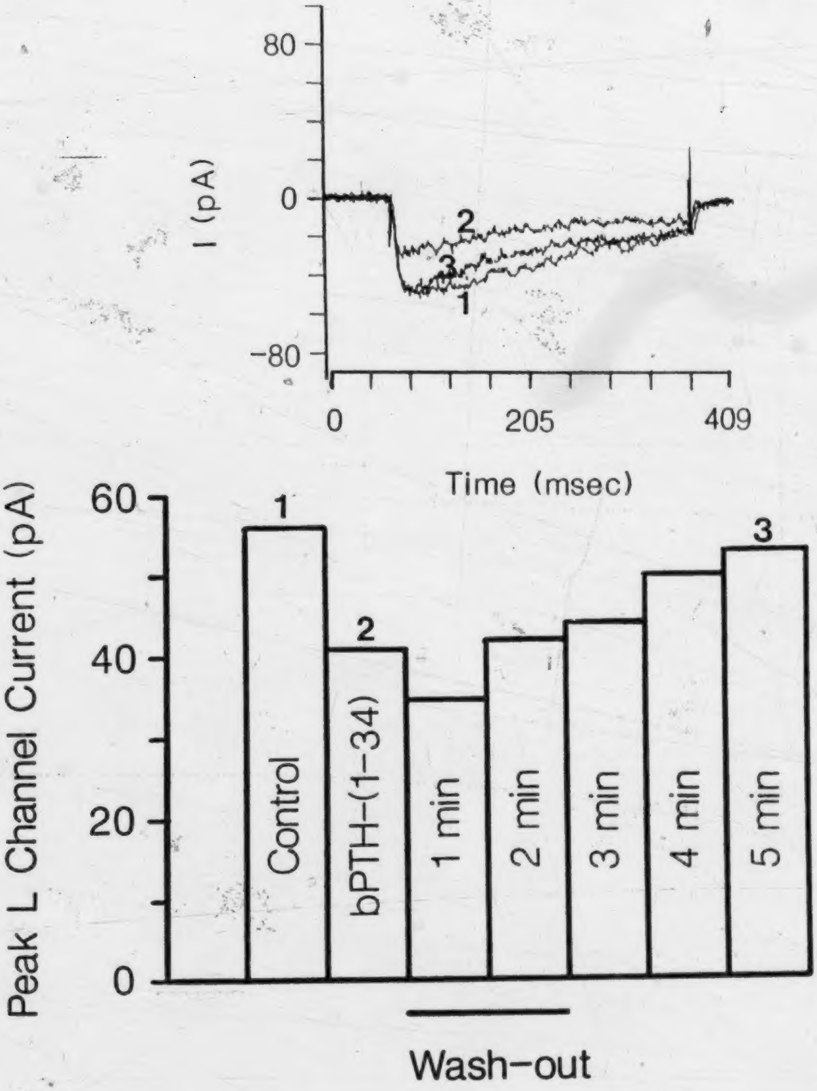

80
0
-80
I (pA)
2
3
1
0
205
409
Time (msec)
60
40
20
0
Peak L Channel Current (pA)
1
2
3
Control
bPTH-(1-34)
1 min
2 min
3 min
4 min
5 min
Wash-out

Fig. VII-10. The reversibility of bPTH-(1-34)-induced inhibition of L channel currents in one vascular smooth muscle cell. bPTH-(1-34) at a concentration of 1 μM inhibited the L channel current by 73%. During the first minute of the wash-out, the peak amplitude of L channel currents further decreased. The wash-out procedure was completed at the end of the two minute period. Then, the amplitude of L channel currents recovered gradually toward the pre-PTH level. The numbers attached to the original current traces (inset) correspond to the different conditions indicated in the histogram. The data are from the same cell.

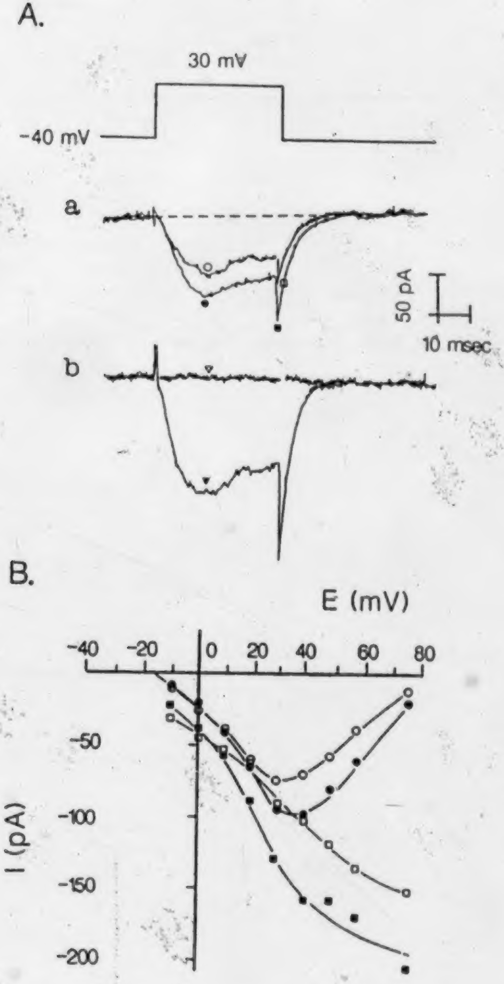

A.
30 mV
-40 mV
a
50 pA
10 msec
b
B.
E (mV)
-40
-20
0
20
40
60
80
I (pA)
-50
-100
-150
-200

Fig. VII-11. The effect of bPTH-(1-34) on the deactivation of L channel currents in one vascular smooth muscle cell. **A.** Tail currents generated from a holding potential of -40 mV to 30 mV. Records in **(A, a-b)** are taken from the same cell. The depolarizing pulse was 40 msec. Leak and capacitative currents have been subtracted. The tail currents have been extrapolated to zero time at which point repolarization was initiated using a single exponential function. Further explanation is found in the text. The tail current (control) had an instantaneous amplitude of 130 pA and a time constant of decay of 5.01 msec. Subsequent application of bPTH-(1-34) decreased the tail current to 92.8 pA with a time constant of 4.91 msec. Bay K-8644 (5 μM) reversed the inhibition of L channel currents by bPTH-(1-34) (solid triangles), with an instantaneous tail current amplitude of 222 pA and a time constant of 3.69 msec. The final application of 2 mM La^{+++} (open triangles) eliminated both the inward current during the test pulse and the tail current. **B.** The I-V relationships for both the peak inward current and the tail current, repolarized to -40 mV from step depolarizations in one cell. The application of bPTH-(1-34) decreased the instantaneous amplitude of tail currents which was proportional to the decrease in the peak inward current. Solid and open circles represent the peak inward current before and after the addition of 1 μM bPTH-(1-34), respectively. Solid and open squares represent the tail current before and after the addition of bPTH-(1-34), respectively.

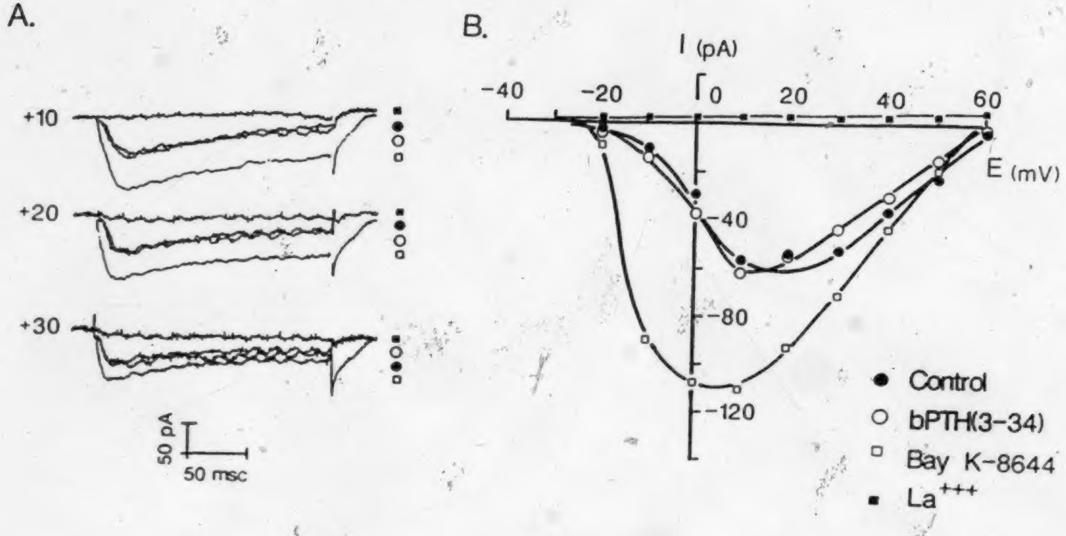

A.
+10
+20
+30
50 pA
50 msc
B.
I (pA)
E (mV)
-40
-20
0
20
40
60
-40
-80
-120
● Control
○ bPTH(3-34)
□ Bay K-8644
■ La+++

Fig. VII-12 The effect of bPTH-(3-34) on L channel currents in a vascular smooth muscle cell. **A.** Original current records at three test pulses. **B.** I-V relationships obtained under different conditions. After a stable recording was obtained (solid circles), 1 μM bPTH-(3-34) was added to the bath (open circles). Five min later, 5 μM Bay K-8644 was applied (open squares) and followed by 2 mM La^{+++} (solid squares). Holding potential: -40 mV. Leak and capacitative currents have been subtracted.

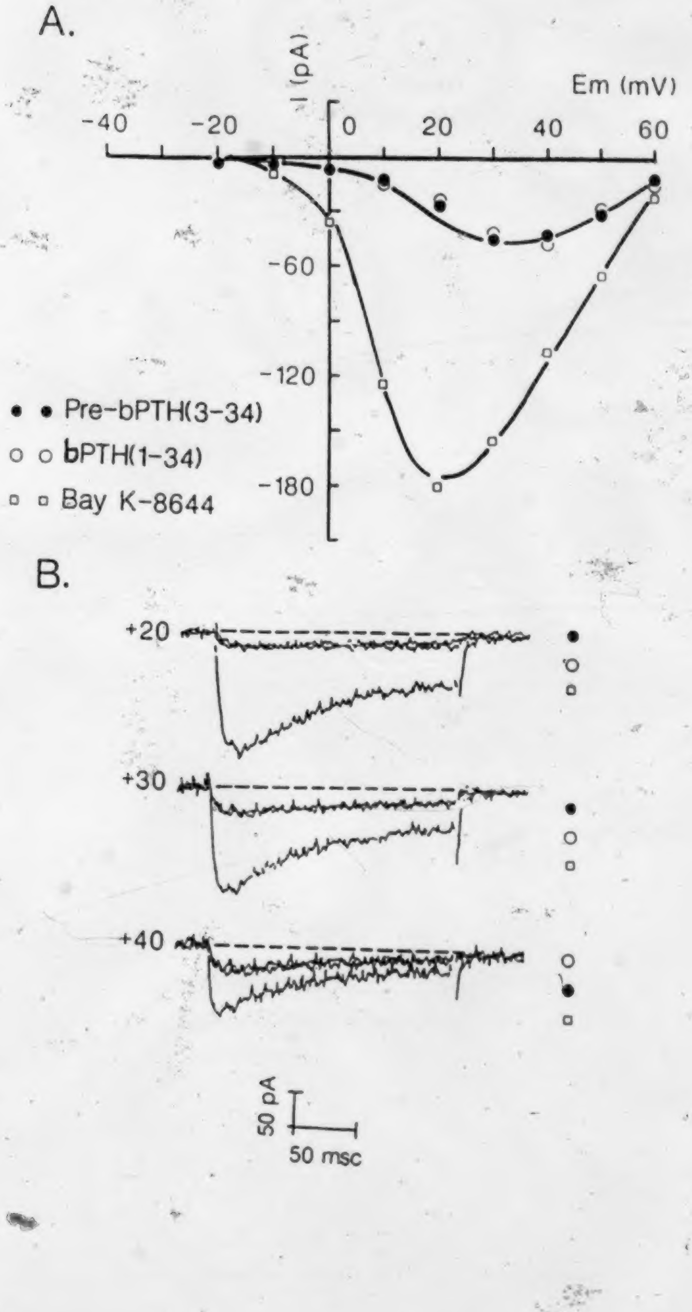

A.
I (pA)
Em (mV)
-40
-20
0
20
40
60
-60
-120
-180
● ● Pre-bPTH(3-34)
○ ○ bPTH(1-34)
▫ ▫ Bay K-8644
B.
+20
+30
+40
50 pA
50 msc

Fig. VII-13 Pretreatment with bPTH-(3-34) abolished the inhibitory effect of bPTH-(1-34) on L channel currents in one vascular smooth muscle cell. **A.** I-V relationships obtained under different experimental conditions. **B.** Original traces with the test potential indicated beside each trace. The cell was pretreated with 1 μM bPTH-(3-34) for 15 min (solid circles) and then exposed to 1 μM bPTH-(1-34) (open circles). Under these conditions, bPTH-(1-34) did not change the L channel current. Subsequent application of 5 μM Bay K-8644 (open squares) increased the inward current. Holding potential: -40 mV. Leakage and capacitative currents have been subtracted.

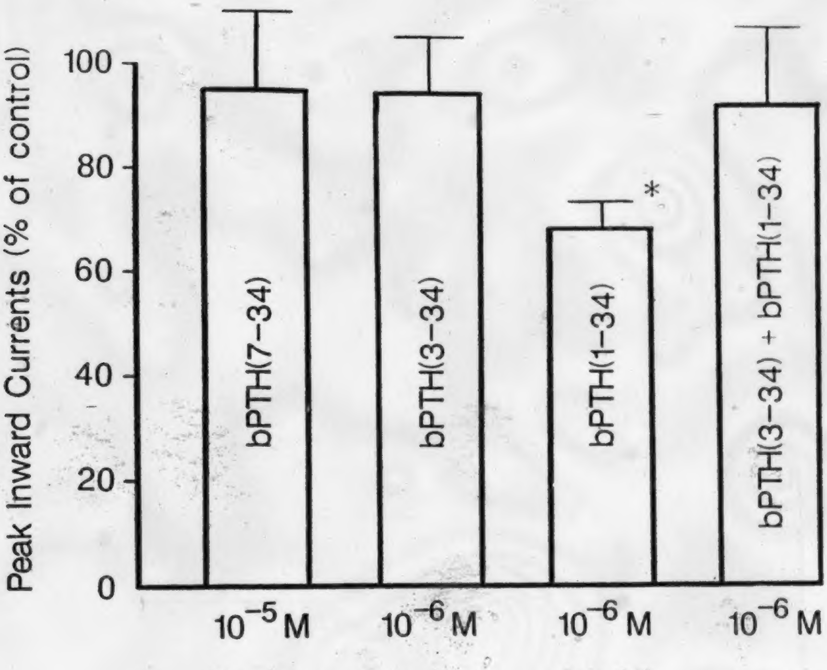

Peak Inward Currents (% of control)
100
80
60
40
20
0
bPTH(7–34)
bPTH(3–34)
bPTH(1–34)
bPTH(3–34) + bPTH(1–34)
*
10^-5 M
10^-6 M
10^-6 M
10^-6 M
(n = 7)
(n = 15)
(n = 20)
(n = 10)

Fig. VII-14. Summary of the effects of different fragments of bPTH on L channel currents in vascular smooth muscle cells. The effects of bPTH fragments were evaluated as the percentage change in L channel currents of the control value. The concentrations of the peptide fragments are indicated in the first row beneath the bar graph. Only bPTH-(1-34) significantly inhibited L channel currents and this inhibitory effect was abolished by pretreatment of cells with bPTH-(3-34). The cell number indicated in the second row includes all cells with or without responses to bPTH fragments. * indicates $p < 0.05$.

CHAPTER 8

The selective effects of PTH fragments on L channel currents in neonatal rat ventricular myocytes

INTRODUCTION

The native parathyroid hormone (PTH) molecule consisting of 1-84 amino acids is present in significant quantities only in the immediate vicinity of the parathyroid gland, while minute amounts are present in the blood plasma (Cohn *et al.*, 1984). A variety of peptide fragments result from cleavage of native PTH either in the gland or in the blood plasma. Most of the biological activities of intact PTH reside in the amino acid sequence 1 to 34, i.e. PTH-(1-34) (Pang *et al.*, 1980a; Rosenblatt, 1986). The cardiac action of bPTH-(1-34) has been studied extensively in recent years. Evidence indicates that PTH possesses both positive chronotropic and inotropic actions (Hashimoto *et al.*, 1981; Tenner *et al.*, 1983). Using isolated spontaneously beating rat right atria, Tenner *et al.* (1983) reported that bovine PTH [bPTH-(1-34)] produced a concentration-dependent increase in beating rate. In addition to bPTH-(1-34), bPTH-(1-84) also produced an immediate and sustained significant increase in beating rate in isolated rat heart cells after 2 days of culture (Bogin *et al.*, 1981). The positive inotropic effect caused by bPTH-(1-34) was abolished by verapamil or a low calcium medium (0.2 mM) (Kondo *et al.*, 1988). Using a single sucrose-gap voltage clamp technique, it was reported that bPTH-(1-34) caused a 37% increase in the peak amplitude of the slow inward calcium channel current in guinea pig papillary muscles, while the outward current was not affected (Kondo *et al.*, 1988). Although this study provided initial evidence that PTH could modulate voltage-gated calcium channels in cardiac muscle, there are some problems which are inherent in the voltage clamp technique when applied to whole tissue preparations, such as nonuniform spatial and temporal distribution of membrane potential, and accumulation of ions in the extracellular space (Johnson and Lieberman, 1971). It is also difficult to rule out possible effects mediated by nerves, neurotransmitters and local reflexes. In view of these considerations, the effect of bPTH-(1-34) on calcium channel currents in single cardiac myocytes needs investigation.

Some fragments of native PTH, such as PTH-(3-34) and PTH-(7-34) act as antagonists of intact PTH in many *in vitro* systems (Huang and Rorstad, 1984; Nickols *et al.*, 1986; Pang *et al.*, 1988a). In cultured opossum kidney (OK) cells (Tamura *et al.*, 1989) and hepatocytes (Mine *et al.*, 1990), bPTH-(3-34) blocked the bPTH-(1-34)-induced transient increase in free intracellular calcium. Although bPTH-(3-34) has no effect on blood pressure (Ellison and McCarron, 1984), bPTH-(3-34) abolished vasodilation induced by bPTH-(1-34) (Huang and Rorstad, 1984; Nickols *et al.*, 1986; Dowe and Joshua, 1987). However, a cardiac action of bPTH-(3-34) has not been reported. The interaction between PTH-(1-34) and PTH-(3-34) in ventricular cells is also unknown.

In order to gain a better understanding of the cardiac action of PTH, the present studies were undertaken. They are as follows: 1) to determine the effects of bPTH-(1-34) and bPTH-(3-34) on the amplitudes of two types of voltage-dependent calcium channels, 2) to compare the effects of these two synthetic fragments of PTH on the kinetics of calcium channel currents, and 3) to determine if bPTH-(3-34) can antagonise the effect of bPTH-(1-34) on calcium channel currents in neonatal rat ventricular myocytes. Using the whole-cell version of the patch clamp technique, the present study directly compares the effects of bPTH-(1-34) and bPTH-(3-34) on the amplitudes and kinetics of both L and T channel currents at the level of single ventricular myocyte.

RESULTS

Effects of bPTH-(1-34) and bPTH-(3-34) on T channel currents:

The amplitude of T channel currents did not change significantly in the presence of 1 μM bPTH-(1-34) (from -159 ±47 pA to -162 ±46 pA, n = 6, P >0.05). When T and L channel currents coexisted in the same cell, the myocyte was first treated with nifedipine to block L channel currents. bPTH-(1-34) was then added to the bath solution and the effect on T channel currents was observed. bPTH-(1-34) had no effect on the residual current (T channel current) after treatment with nifedipine. One example is shown in Fig. VIII-1.

Another experimental protocol to study the effect of PTH fragments on T channel currents is shown in Fig. VIII-2. Pretreatment of the cell with 10 μM nifedipine for 10 min completely eliminated L channel currents. Subsequent application of 1 μM bPTH-(1-34) for 15 min did not change the amplitude or the I-V relationship of T channel currents. This experiment was repeated in another 4 cells. Concentrations of bPTH-(1-34) ranging from 0.1 to 10 μM failed to modulate the T channel current.

In addition, the peak amplitude of T channel currents did not change significantly in the presence of 1 μM bPTH-(3-34). When the amplitudes of T channel currents were compared before (-168 ±78 pA) and after (-154 ±76 pA) the application of 1 μM bPTH-(3-34), no significant change could be detected (n = 6, p > 0.05). Subsequent application of Bay K-8644 also failed to affect these T channel currents, indicating insensitivity to dihydropyridines.

The effect of bPTH-(1-34) on the amplitude of L channel currents:

Usually, a period of 1 to 5 min was required for either Bay K-8644 or nifedipine to exert a maximum effect on L channel currents. However, a longer time, ranging from 5 to 15 min, was needed for PTH to modulate the inward current in neonatal rat ventricular myocytes. The effect of bPTH-(1-34) on L channel currents in one cell is shown in Fig. VIII-3. bPTH-(1-34) at a concentration of 1 μM increased the amplitude of L channel currents by 58% in this cell.

The concentration-dependent effect of bPTH-(1-34) on L channel currents in ventricular myocytes is shown in Fig. VIII-4. The lowest effective concentration of bPTH-(1-34) was 0.01 μM, which increased the L channel currents by 19.4 ± 5% ($p<0.05$). A concentration of 0.1 μM bPTH-(1-34) increased the L channel currents by 24.5 ± 6.3% ($p<0.05$). The maximum increase in the amplitude of L channel currents was seen in the presence of 1 μM bPTH-(1-34) (73 ±13% increase, $p<0.05$). When the concentration of bPTH-(1-34) was increased to 3 μM, it failed to increase L channel currents any further (31.7 ± 13% increase). In fact, bPTH-(1-34) at a concentration of 3 μM showed a lesser effect compared with that of 1 μM.

The normalized conductance of L channels in the absence or presence of bPTH-(1-34) is shown in Fig. VIII-5. The conductance was calculated from the equation: $G_{(L)} = I_{(L)} / (V_T - V_{rev})$. The apparent reversal potential of L channels is determined as the intersection of the I-V curves obtained before and after the application of 2 mM La^{+++} in the bath. V_T is the test pulse at which the L channel current, $I_{(L)}$, is recorded. Since the peak inward current during a test pulse is used as the indication of $I_{(L)}$, this may underestimate the absolute peak amplitude of I_L due to the slow inactivation of L channel currents. The maximum conductance is taken as 1.00. In the presence of 1 μM bPTH-(1-34), the normalized conductance curve for L channels was shifted to the left by approximately 10 mV, indicating that the maximum conductance for L channels could be obtained at lower depolarizations in the presence of bPTH-(1-34). The steady-state inactivation of L channel currents in the presence of bPTH-(1-34) was further examined. The holding potential was changed in a stepwise manner with a fixed depolarizing pulse to +20 mV. The membrane potential was first conditioned for 3 sec to reach a quasi-steady state and a test pulse of 320 ms then followed. The peak current amplitudes during the test pulses were measured at each holding potential and normalized to the maximum peak amplitude of inward currents. bPTH-(1-34) at 1 μM did not shift the steady-state inactivation curve (Fig. VIII-6 b) although the absolute amplitude of L channel currents was enhanced (Fig. VIII-6 a). This result indicates that the effect of bPTH-(1-34) on the amplitude of L channel currents was not dependent on the holding potential.

To test the reversibility of the effect of bPTH-(1-34), a "wash-out" procedure was carried out after the increase in L channel currents induced by bPTH-(1-34) was established. Satisfactory replacement of the bath solution was obtained with a perfusion rate of approximately 1 ml/10 sec. As shown in Fig. VIII-7, it was difficult to reverse the excitatory effect of bPTH-(1-34) on L channel currents although the kinetics of inactivation of L channel currents were changed after the "wash-out". The amplitudes of L channel currents were almost the same before and after a 2 min wash-out and 3 min of recovery, suggesting that either 5 min was too short to wash away bPTH-(1-34) from its binding site on the ventricular myocyte membrane or the phosphorylation of cardiac L channels induced by bPTH-(1-34) was not easily reversed. The wash-out resistant actions of endothelin on calcium channels in vascular smooth muscle cells (Inoue *et al.*, 1990), 8-bromo-cyclic GMP on L channel currents in embryonic ventricular myocytes (Wahler *et al.*, 1990) and dopamine on T-channel currents in neurons (Carbon and Lux, 1989) have also been reported. In contrast, the inhibitory effect of bPTH-(1-34) on L channel currents in vascular smooth muscle cells was reversible (Chapter 7). These results suggested that different intracellular mechanisms probably mediated the effect of bPTH-(1-34) on L channel currents in the two different tissues.

The effect of bPTH-(3-34) on the amplitude of L channel currents:

At a concentration of 1 μM, bPTH-(3-34) failed to affect the amplitude of L channel currents, as shown in Fig. VIII-8. After the control records were obtained, subsequent application of bPTH-(3-34) did not change the amplitude, the threshold potential at which the L channel was activated, the peak potential at which the currents reached their maximum value or the reversal potential. After the addition of bPTH-(3-34) into the bath for 15 min, 5 μM Bay K-8644 was applied to the same cell. Bay K-8644 increased the amplitude and shifted the peak of L channel currents toward a more negative potential, which further confirmed that the channel current under investigation was of the L type. This also indicated that bPTH-(3-34) did not affect the action of Bay K-8644 on L channels.

To further exclude the effect of the time-dependent deterioration, i.e. "run-down", of calcium channel currents on the analysis of data, the time-dependent changes of T and L channel currents in the presence or absence of 1 μM bPTH-(3-34) were compared (Fig. VIII-9). Within 20 min, the "run-down" of both T and L channel currents were less than 20% of the control value taken at the beginning of the recording. During this period of time, no significant changes in the amplitudes of either T or L channel currents could be found before and after the application of 1 μM bPTH-(3-34).

In another group of experiments (Fig. VIII-10), single ventricular cells were pretreated with 1 μM bPTH-(3-34) for more than 15 min. The peak amplitude of L channel currents was then recorded as the control value (100 ±13%). 1 μM bPTH-(1-34) was subsequently applied to the cell, and the relative peak amplitude of L channel currents was 92 ±14% ($n=5$, $p>0.05$). Under these conditions, the effect of bPTH-(1-34) on L channel currents, i.e. amplification, was abolished by bPTH-(3-34) pretreatment at an equimolar concentration.

Effects of bPTH-(1-34) and bPTH-(3-34) on the kinetics of L channel currents

A calcium channel antagonist or agonist can alter the kinetics of L channel currents by changing the calcium channel gating mode. In the present study, it was found that Bay K-8644 at a concentration of 5 μM shortened the time courses of both the activation and inactivation of L channel currents, as determined by the time constants of activation and inactivation. These results are shown in Table VIII-1 and Fig. VIII-11C.

The time constant of the inactivation of L channel currents was the same before and after the application of 1 μM bPTH-(1-34). However, bPTH-(1-34) prolonged the time course of the activation of L channel currents. The time constant was changed from 1.81 msec to 2.49 msec (Fig. VIII-11A and Table VIII-1).

In contrast, bPTH-(3-34) changed the kinetics of inactivation, but not that of the activation, of L channel currents. A set of L channel current traces before and after the application of bPTH-(3-34) is shown in Fig. VIII-11B. The time constant of the inactivation of L channel currents was 232 msec before and 116 msec after application of 1 μM bPTH-(3-34). With increased inactivation, the amplitude of L channel currents was even smaller than the control trace at the end of the depolarization pulse. Fig. VIII-12 shows the time-related changes in the time constants of L channel current inactivation at two different membrane potentials. Under control conditions, i.e. in the absence of PTH fragments, the time constant of the inactivation of L channel currents did not change within 15 min. After the application of 1 μM bPTH-(3-34), the time constant of inactivation gradually decreased. This decrease became significant at 10 min ($p<0.05$) and even more obvious at 15 min.

Interaction of bPTH-(1-34) and dihydropyridines:

As shown in Fig. VIII-4, the maximum increase in L channel currents caused by bPTH-(1-34) was obtained when the concentration of the peptide reached 1 μM. However, this augmentation of L channel currents by 1 μM bPTH-(1-34) could be further enhanced by Bay K-8644 (Fig. VIII-13). The rate of inactivation was increased and the I-V relationship shifted toward more negative potentials. In 5 cells, Bay K-8644 at 5 μM elicited an additional increase in L channel currents (264 ±51%) compared with the previous augmentation of L channel currents by 1 μM bPTH-(1-34) ($p<0.05$). The increase in the amplitude of L channel currents by Bay K-8644 (5 μM) under two conditions, i.e. in the presence or absence of bPTH-(1-34), showed no statistically significant difference ($p>0.05$) (Fig. VIII-14). It would appear that, as expected, bPTH-(1-34) and Bay K-8644 work via independent mechanisms.

In the next group of cells, the interaction of bPTH-(1-34) and nifedipine, an L channel antagonist, was tested. L channel currents that had been enhanced by bPTH-(1-34) were subsequently inhibited by nifedipine (10 μM), as shown in Fig.

VIII-15. On the other hand, the L channel currents inhibited by nifedipine were amplified by bPTH-(1-34), as shown in Fig. VIII-1.

DISCUSSION

PTH is a selective activator of L channels in ventricular cells.

Voltage- and time-dependent calcium channels in cardiac myocytes may be modulated by some endogenous peptides. Using the whole-cell version of the patch clamp technique, Ono *et al.* (1989) reported that calcitonin gene-related peptide enhanced calcium currents (I_{Ca}) in frog and rabbit atrial myocytes. Callewaert *et al.* (1989) observed the enhancement of L channel currents in guinea pig ventricular myocytes by a low molecular weight endogenous peptide isolated and purified from rat brain. In the present study, direct electrophysiological evidence demonstrates that PTH, an endogenous circulating hormone, enhances L channel currents in ventricular myocytes. This excitatory effect of bPTH-(1-34) on L channels needed more than 5 min to fully develop and was not reversible. The latency of onset of PTH effects may reflect the involvement of intracellular second messengers. As mentioned in Chapter 2, the intracellular free calcium concentration is less than 10^{-9} M in the present patch clamp studies. If the dephosphorylation of L channels in neonatal rat ventricular myocytes is intracellular calcium dependent, the rate of dephosphorylation of L channels will be very slow in the presence of a low intracellular free calcium concentration. This mechanism may explain the relative irreversibility of the PTH effect. Alternatively, a 2 min wash-out may not effectively wash away PTH from its binding site. Further discussion of the difference in PTH effects on L channel currents in vascular smooth muscle cells and ventricular myocytes is found in Chapter 11. In addition, PTH did not affect T channel currents in ventricular myocytes. This is the first report to show the selective effect of PTH on two types of voltage-dependent calcium channel currents in isolated heart cells, using the patch clamp technique. The modulation of calcium channels in cardiac myocytes by PTH explains one of the mechanisms underlying the cardiac effects of PTH. For example, the positive inotropic effect of PTH (Katoh *et al.*, 1981; Kondo *et al.*, 1988) may be explained by results from the present patch clamp studies. The augmentation of L channel currents by PTH will inject Ca^{++} into the cytosol to support the strengthened contraction of cardiac muscle. The effects of PTH on other ion channels, such as K^+ and Na^+ channels, and receptor-operated channels in ventricular cells are unknown. However, the positive chronotropic effect of PTH could still be partially accounted for by the PTH effect on L channels. By increasing the amplitude of L channel currents, PTH may increase the spontaneous rate of action potential generation. In the presence of bPTH-(1-34), the normalized conductance curve shifts toward more negative potentials and, therefore, L channels will activate at more negative potentials. *In vivo*, this would increase the rate of the upstroke phase (Strauss *et al.*, 1977; Irisawa *et al.*, 1978) and the later phase of diastolic depolarization of the cardiac pacemaker tissue (Noma *et al.*, 1980), leading to an increase in the beating rate. Similar mechanisms for the positive chronotropic effect of epinephrine have also been described (West *et al.*, 1956).

Does PTH act on L channels in the same way as Bay K-8644 does?

The rationale for studying the interaction of the effects of PTH and Bay K-8644 on calcium channel currents has been stated in Chapter 7. The data presented in this thesis indicate that PTH and Bay K-8644 have similar effects on L channel currents in ventricular cells. Both increased the amplitude of L channel currents without producing any effect on T channel currents. The effect of bPTH-(1-34) and Bay K-8644 on L channel currents in neonatal rat ventricular cells could be antagonized by nifedipine. Furthermore, the normalized conductance or activation curves were shifted along the voltage axis toward more negative potentials in the presence of either bPTH-(1-34) or Bay K-8644. Bay K-8644 also shifted the activation curve of L channels in adrenal medullary chromaffin cells (Cena *et al.*, 1989), rat tail artery smooth muscle cells (Wang *et al.*, 1989) and rat ventricular cells (Sanguinetti *et al.*, 1986). However, this similarity does not imply an identical mechanism. The differences in the effects of these two agents on L channels were evident. 1). The effects of bPTH-(1-34) and Bay K-8644 were synergistic. 2). Bay

K-8644 elicited a reaction in almost every ventricular cell, while bPTH-(1-34) only acted on a percentage of cells. 3). In different types of cells, the effect of PTH on L channel currents was different, such as amplification in cardiac myocytes but inhibition in neuroblastoma cells (Pang *et al.*, 1990). However, Bay K-8644 always activated L channels in various cell preparations. 4). It took 5 to 15 min for PTH to activate L channels maximally but only 1 to 7 min was needed for Bay K-8644 in neonatal rat ventricular myocytes. 5). Bay K-8644 is permeant to the cell membrane while PTH may be not.

Considering the similarities and differences between the effects of bPTH-(1-34) and Bay K-8644, it is suggested that the mechanism by which PTH amplified L channel currents is distinct from that responsible for the effect of Bay K-8644. It has been reported that the activation of membrane bound G proteins by Bay K-8644, and other dihydropyridines, directly modulates L channels (Scott *et al.*, 1988; Bergamaschi *et al.*, 1990). Furthermore, it is not likely that cAMP-dependent protein phosphorylation would be coupled to the effect of Bay K-8644 on L channel currents (Hess *et al.*, 1984). From the data presented here, little can be inferred about the mechanisms of PTH action on L channel currents. However, several possibilities exist. PTH may act directly on the protein complex of calcium channels. Alternatively, the effect of PTH could be secondary to the change in some membrane transducers or intracellular second messengers. The activation of cAMP-dependent phosphorylation by PTH has been suggested to be responsible for the mediation of PTH-elicited calcium mobilization (Bogin *et al.*, 1981; Tenner *et al.*, 1983; Kondo *et al.*, 1988). On the other hand, the increase in the intracellular level of cAMP has been demonstrated to increase calcium channel currents in frog ventricular or atrial myocytes (Ono *et al.*, 1989; Schouten *et al.*, 1989). In the present study, the relatively long time needed for the onset of the PTH effect on L channel currents also suggests the involvement of a second messenger(s). This difference in the intracellular mediating mechanisms for PTH and Bay K-8644 might be able to explain for their different actions on calcium channels. This hypothesis is confirmed by the results presented in Chapter 10, in which the cAMP mediated PTH effect on L channel currents is demonstrated. However, the effect of Bay K-8644 is not dependent on the activation of cAMP-dependent protein kinase (see Chapter 10).

Tachyphylaxis of PTH effect or multiple subtypes of PTH receptors?

PTH-elicited cardiovascular effects are concentration dependent. The limited functional concentration range of bPTH-(1-34), at which the peptide activated L channel currents, is not unexpected. At a higher concentration, not only peptides (Huang BS, *et al.*, 1987) but also Bay K-8644 (Su *et al.*, 1984) exhibited self-inhibition. When the concentration of PTH was increased beyond a critical value, the effect of PTH was decreased, rather than continuously increased or saturated. A similar pattern can be found in the dose-response relationship for PTH-induced inositol phosphate production in isolated hepatocytes (Mine *et al.*, 1989), in the vasodilator action of PTH on isolated perfused rat kidney (Musso *et al.*, 1989) and in the force development in rat papillary muscles induced by various concentrations of PTH (Katoh *et al.*, 1981). In the present study with neonatal rat ventricular myocytes, the maximum amplification of L channel currents was achieved by 1 μM bPTH-(1-34). PTH concentrations above 1 μM produced only a sub-maximal increase in the amplitude of L channel currents. The explanation for this concentration-related action of PTH is not obvious. Musso *et al.* (1989) postulated that the tissue became rapidly resistant to PTH, and so exhibited a decreased response to the high concentration of PTH. However, this tachyphylaxis was not observed in rats and dogs (Pang *et al.*, 1980a, b). In some neonatal rat ventricular myocytes, a transient increase, and subsequent decrease, in the amplitude of L channel currents was observed following exposure to a single high concentration of bPTH-(1-34). This result may indicate the mechanisms of tachyphylaxis. However, more evidence is needed to answer this question satisfactorily.

The complexity of receptor subtypes for families of endogenous substances is known. For example, the existence of at least two subtypes of vasopressin receptors has been demonstrated (Fishman *et al.*, 1987; Marchingo *et al.*, 1988). These two subtypes of vasopressin receptors have different distributions, second messengers, and ligand specificity. Different responses of various tissues to PTH could also be related to the existence of multiple subtypes of PTH receptors. Two subtypes of

PTH receptors, as reported in thymocytes (Hesch *et al.*, 1986), may provide an alternate explanation for the concentration-related effect of PTH in the present study. At lower concentrations, bPTH-(1-34) might bind to a P_a receptor, which could either be part of the L channel protein or be located near it and, hence, result in an increase in the channel currents. At higher concentrations, bPTH-(1-34) might bind to a P_i receptor in addition to P_a. The activation of the P_i receptor could cause an inhibition of L channels. The net result would be determined by the proportion of the occupancy of the two types of PTH receptors.

PTH has a different effect on L-type voltage-dependent calcium channels in various cell types. This hormone inhibited L channel currents in neuroblastoma cells and vascular smooth muscle cells (Pang *et al.*, 1990) while it activated L channels in ventricular cells. The tissue-selective distribution of the two types of PTH receptors is one of the possible mechanism underlying the differential actions of PTH on L channels in various tissues. Further electrophysiological and biochemical evidence is needed to corroborate this hypothesis.

Different effects of PTH fragments on L channel currents in ventricular myocytes determined by amino acid sequence

In the present studies, direct electrophysiological evidence indicated that bPTH-(1-34) and bPTH-(3-34) had different actions on calcium channels in ventricular myocytes. Both bPTH-(1-34) and bPTH-(3-34) had no effects on the amplitude or kinetics of T channel currents. bPTH-(1-34), but not bPTH-(3-34), increased the amplitude of L channel currents in neonatal rat ventricular cells. Both bPTH-(1-34) and bPTH-(3-34) modified the kinetics of L channel currents in this cell preparation. bPTH-(3-34) affected L channel currents by changing the kinetics of the inactivation, whereas bPTH-(1-34) changed the kinetics of the activation of L channel currents. In terms of receptor binding of PTH, an electrophysiological approach is used to show that bPTH-(1-34) and bPTH-(3-34) possibly act on the same binding site and, in turn, affect the L channel current, directly or indirectly. This speculation is drawn from the fact that pretreatment of cells with bPTH-(3-34) abolished the effect of bPTH-(1-34) on both the amplitude and kinetics of L channel currents.

The modification of L channel current activation kinetics by dopamine (Carbone and Lux, 1989), enkephalin (Tsunoo *et al.*, 1986) and noradrenaline (Brown *et al.*, 1989) have been reported in neurons. Tsunoo *et al.* (1986) suggested that the slowness of activation kinetics was due to the binding of agents to the "resting state" of calcium channels. Furthermore, effects of opiates and noradrenaline on L channel currents, including the modification of kinetics, may be mediated by a G-protein (Hescheler *et al.*, 1987b; Brown *et al.*, 1989). It has also been indicated that a G-protein acts as a coupling factor between PTH and the catalytic subunit of adenylate cyclase (Teitelbaum *et al.*, 1982). Whether a G protein is responsible for the effect of bPTH-(1-34) on the activation kinetics of L channel currents needs further investigation.

The increase in the inactivation rate of L channel currents in the presence of bPTH-(3-34) could be attributed to blockage of the open state of L channels (Colquhoun and Hawkes, 1983; Hering *et al.*, 1988). The obvious modification of inactivation kinetics by bPTH-(3-34) suggested that the Ca^{++} channel gating mode was changed, as is the case with dihydropyridines and L channel currents (Colquhoun and Hawkes, 1983; Hering *et al.*, 1988). Hess *et al.* (1984) related the decay of L channel currents to the enhancement of gating mode 2 by Bay K-8644. To determine whether this is the same mechanism responsible for the effect of bPTH-(3-34) on L channel currents in neonatal rat ventricular cells will require direct evidence from recording and analysis of the single channel currents.

In neonatal rat ventricular muscle, whether bPTH-(3-34) antagonizes the effect of bPTH-(1-34) on muscle contraction is not known. A higher concentration of bPTH-(3-34) may be required to abolish the effect of bPTH-(1-34) on cardiac muscle contraction if an analogy is made from the study of smooth muscle (see discussion of Chapter 7). However, in the present study, bPTH-(3-34) at an equimolar concentration totally abolished the effect of bPTH-(1-34) on L channel currents. This result was not unexpected in terms of the concentration used since here a specific type of calcium channel current rather than cell contraction is concerned. Contraction of myocytes depends on multiple factors, of which Ca^{++} influx through voltage-dependent channels is only one. Moreover, the modification of voltage-dependent calcium channels is not the only action of PTH. PTH can also act on the production of cAMP (Nickols *et al.*, 1987) and inositol phosphates (Coleman and Bilezikian, 1990) as well as the Ca^{++}-extrusion pump [(Ca^{++}-Mg^{++})-ATP_{ase}] (McKenzie *et al.*, 1990) in various cell preparations. These effects of PTH may also contribute to muscle contraction. On the whole, it is understandable that an equimolar concentration of bPTH-(3-34) could not abolish muscle contraction, but did affect the increase in L channel currents induced by bPTH-(1-34). The relationship of cAMP content and L channel activity in the presence of PTH is not clear but is a very interesting topic. If the PTH effect on calcium channels in ventricular myocytes is mediated by cAMP, the modification of L channel currents, then, needs a threshold concentration of cAMP. Partial inhibition of the bPTH-(1-34) effect on cAMP concentrations by bPTH-(3-34) might be enough to keep the intracellular concentration of cAMP below the threshold level, and, consequently, eliminate the effect of bPTH-(1-34) on L channel currents.

Under pathophysiological conditions such as uremia (Jüppner and Hesch, 1982), various fragments of PTH appeared in abnormal amounts in the peripheral circulation. It is believed that these PTH peptides act on the different subtypes of PTH receptors in different tissues (Hesch *et al.*, 1986). N-terminal-deleted PTH peptides, such as PTH-(3-34) and PTH-(7-34), activate type-II PTH receptors and antagonise the classical type-I PTH receptors which are only sensitive to the intact N-terminal PTH peptide, such as PTH-(1-34) (Hesch *et al.*, 1986). This hypothesis of two PTH receptors has been used to explain the deleterious effects induced by high circulating levels of PTH peptides on various tissues, including the cardiovascular system. If the circulating N-terminal-deleted PTH peptides increased abnormally, such as in uremia (Massry, 1987), the binding of these peptides would affect L-type voltage-dependent calcium channels in such a way that the effects of the intact N-terminal PTH peptides are inhibited. The effects of bPTH-(1-34) on L channels is inhibitory in smooth muscle and neuronal cells (Pang *et al.*, 1990), and excitatory in ventricular cells. The consequence of the inhibition of the bPTH-(1-34) effect under specific pathophysiological conditions would be, therefore, the cancellation of the bPTH-(1-34) effect on calcium channels in various tissues. Calcium overload in vascular smooth muscle and chronic cardiac failure would be expected, as is the case in hypertension (Bruschi *et al.*, 1985; Sugiyama *et al.*, 1990).

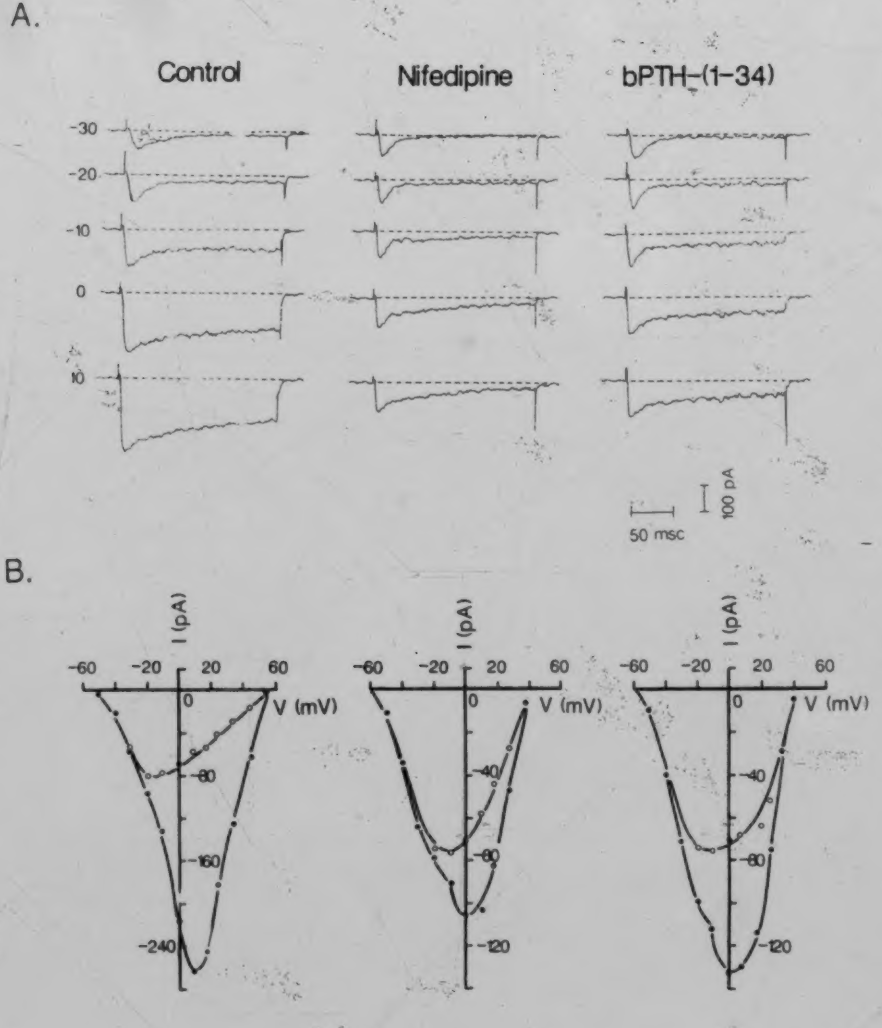

A.
Control
Nifedipine
bPTH-(1-34)
-30
-20
-10
0
10
100 pA
50 msc
B.
I (pA)
V (mV)
-60
-20
0
20
60
-80
-160
-240
-40
-80
-120

Fig. VIII-1. The effects of nifedipine and bPTH-(1-34) on T channel currents in ventricular myocytes. **A.** Depolarization to different membrane potentials, indicated in mV beside each trace, from a holding potential of -80 mV elicited both T and L channel currents. **B.** The I-V relationships of the inward currents under different conditions. Total currents (filled circles) were measured as the peak values of inward currents. The non-inactivated component measured at the end of 200 ms depolarization is an indication of the L channel component. Subtraction of this L component from total currents yielded a residual component (open circles), which is a relative measure of the T channel component. This assumes that the L channel component does not inactivate appreciably. The left panel shows the control recording. The middle panel shows the recordings in the presence of 1 μM nifedipine. Most of the L channel currents were blocked by nifedipine while the T channel currents remained. The right panel shows that subsequent application of 1 μM bPTH-(1-34) did not change the T channel currents but increased the L channel currents. The T channel currents were the same under these three conditions. Leakage and capacitive currents have been subtracted. The dashed lines represent the zero current level.

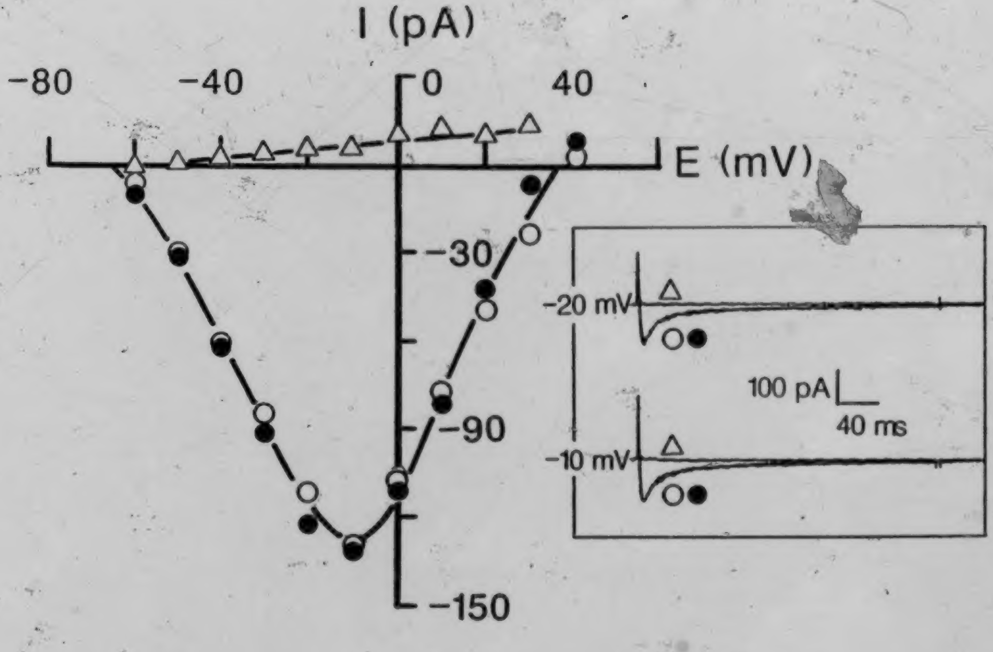

I (pA)
−80
−40
0
40
E (mV)
−30
−90
−150
−20 mV
△
○ ●
100 pA
40 ms
−10 mV
△
○ ●

Fig. VIII-2. The response of T channel currents to bPTH-(1-34) in one ventricular myocyte. The single ventricular myocyte was pretreated with 10 μM nifedipine for 10 min. After control currents (open circles) were recorded, 1 μM bPTH-(1-34) was applied to the same cell and the inward currents were recorded (filled circles). The subsequent application of 2 mM La^{+++} eliminated the transient inward currents (open triangles). The original T channel current traces at two membrane potentials are shown in the inset. Leakage and capacitive currents have been subtracted from the original channel current traces.

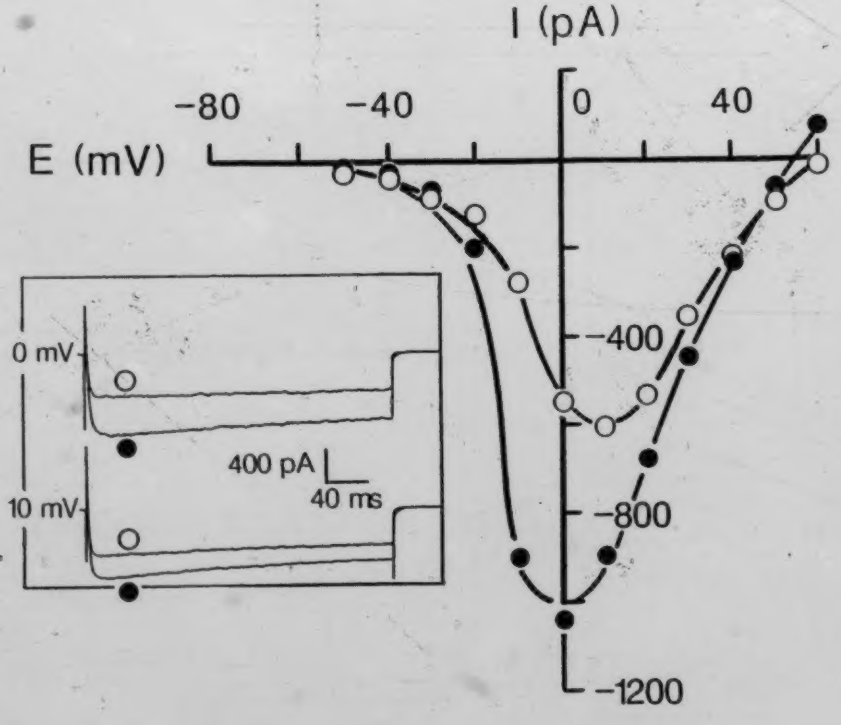

I (pA)
E (mV)
−80
−40
0
40
−400
−800
−1200
0 mV
10 mV
400 pA
40 ms

Fig. VIII-3. The effect of bPTH-(1-34) on L channel currents in neonatal rat ventricular cells. After control currents (open circles) were recorded, 1 μM bPTH-(1-34) was applied to the same cell and the inward currents were recorded (filled circles). Leakage and capacitive currents have been subtracted from the original current traces. The holding potential was set at -80 mV. The L channel current traces at two depolarization levels are shown in the inset.

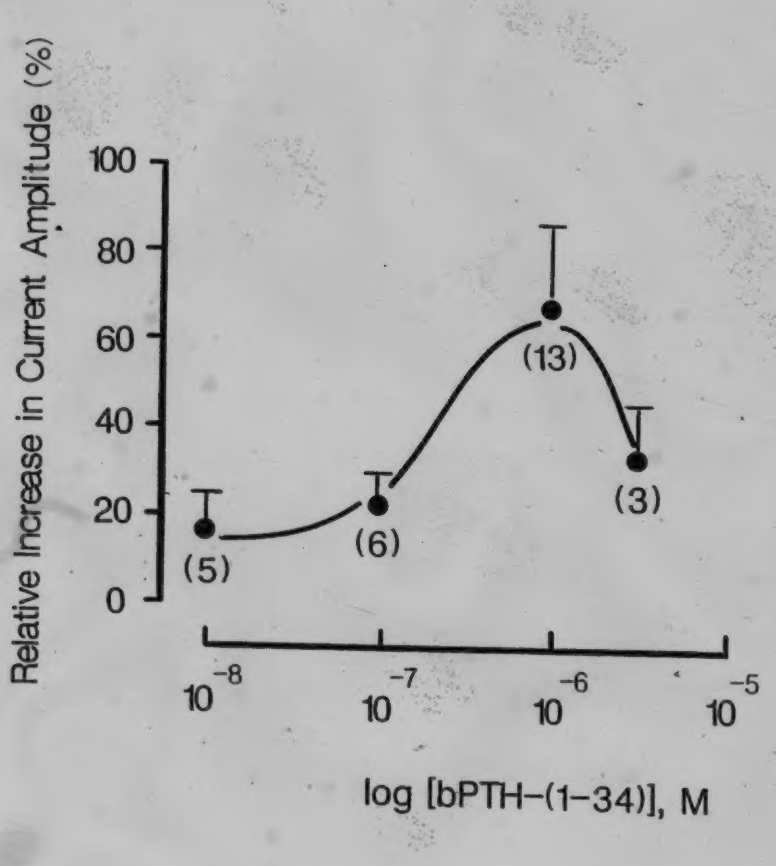

Relative Increase in Current Amplitude (%)
100
80
60
40
20
0
(5)
(6)
(13)
(3)
10^{-8}
10^{-7}
10^{-6}
10^{-5}
log [bPTH-(1-34)], M

Fig. VIII-4. Concentration-related responses of L channel currents to bPTH-(1-34) in ventricular cells. bPTH-(1-34) increased L channel currents in a concentration-dependent manner. The amplitude of L channel currents in the absence of bPTH-(1-34) was taken as 100%. bPTH-(1-34) significantly increased L channel currents at all concentrations tested ($p<0.05$). At 10^{-8} M, bPTH-(1-34) increased L channel currents by 19±5%. The saturated increase in the amplitude of L channel currents was reached at 1 μM bPTH-(1-34). As the concentration increased above 1 μM, bPTH-(1-34) induced less increment in the L channel current. The number of cells tested at a particular concentration is shown at every point on the curve. The solid curve was fitted by eye.

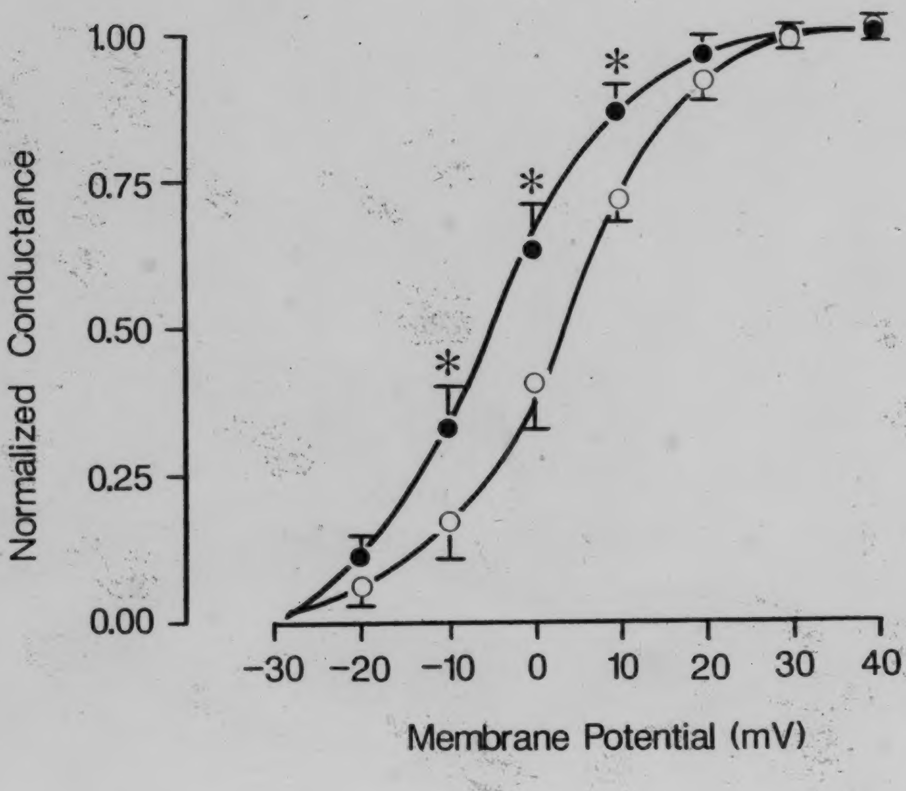

Normalized Conductance
1.00
0.75
0.50
0.25
0.00
*
*
*
−30
−20
−10
0
10
20
30
40
Membrane Potential (mV)

Fig. VIII-5. The normalized conductance curves for L channels in the absence or presence of 1 μM bPTH-(1-34). In the absence of bPTH-(1-34) (open circles), maximum and half-maximum conductances for L channels were achieved at +30 mV and +3 mV, respectively. After treatment with bPTH-(1-34) (filled circles), the maximum and half-maximum conductances for L channels were obtained at +20 and -4 mV, respectively. The conductance vs voltage curve shifted toward the direction of negative potentials in the presence of bPTH-(1-34). The data points on the curves are plotted as mean ± SE, $n=5$. * indicates $p<0.05$ when the corresponding points on two curves were compared. The solid curves were fitted by eye.

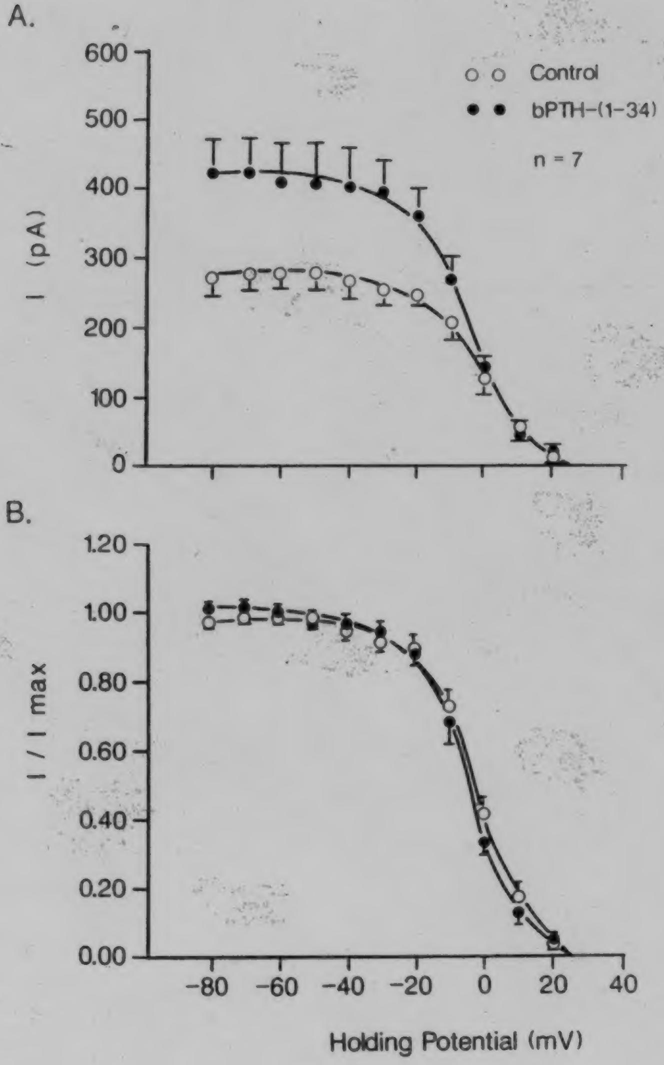
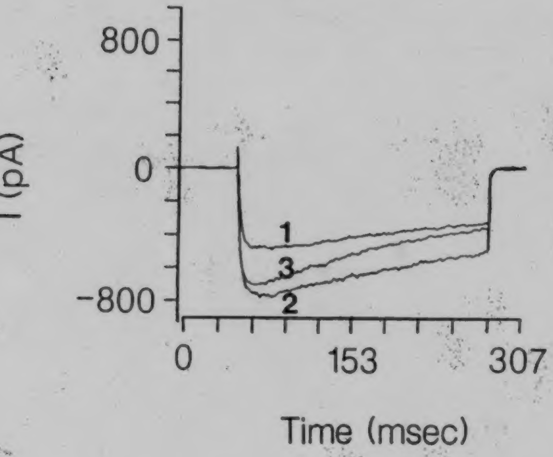
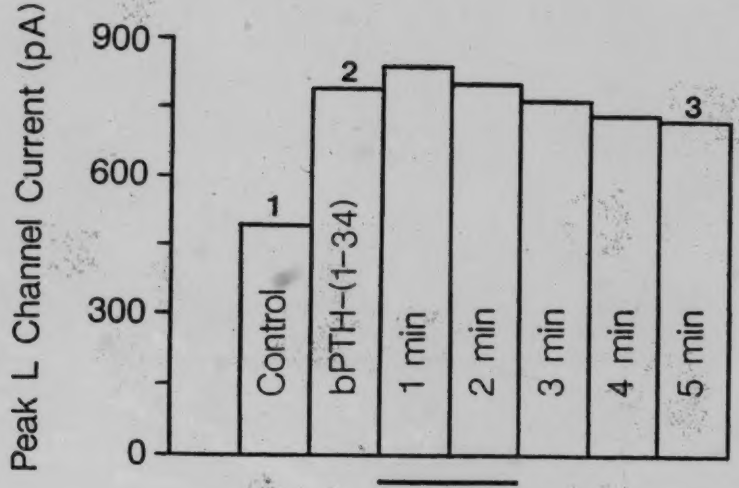

A.
600
500
400
300
200
100
0
I (pA)
○ ○ Control
● ● bPTH-(1-34)
n = 7
B.
1.20
1.00
0.80
0.60
0.40
0.20
0.00
I / I max
-80
-60
-40
-20
0
20
40
Holding Potential (mV)

Fig. VIII-6. The steady-state inactivation of L channel currents in response to 1 μM bPTH-(1-34) in ventricular myocytes. The holding potential was changed in a stepwise manner with a fixed depolarizing pulse to +20 mV. **A.** The absolute amplitudes of L channel currents were significantly enhanced by bPTH-(1-34) at holding potentials more negative than -10 mV ($p<0.05$). **B.** The relative amplitudes of L channel currents at all the holding potentials examined and the slopes of the steady-state inactivation curves did not change in the absence or presence of bPTH-(1-34) ($p>0.05$).


800
0
−800
I (pA)
1
3
2
0
153
307
Time (msec)


Peak L Channel Current (pA)
900
600
300
0
1
2
3
Control
bPTH-(1-34)
1 min
2 min
3 min
4 min
5 min
Wash-out

7

Fig. VIII-7. The reversibility of the bPTH-(1-34)-induced increase in L channel currents in one ventricular myocyte. At a concentration of 1 μM, bPTH-(1-34) increased the amplitude of L channel currents by 161%. During the first minute of wash-out, the amplitude of L channel currents increased continuously. The wash-out procedure was completed at the end of the two minute period. At 5 minute after the beginning of the wash-out, the amplitude of L channel currents was still 48% higher than the control value. The excitation effect of bPTH-(1-34) on L channel currents was not reversed significantly within 5 min. The numbers attached to the original current traces (inset) correspond to the different conditions indicated in the histogram.

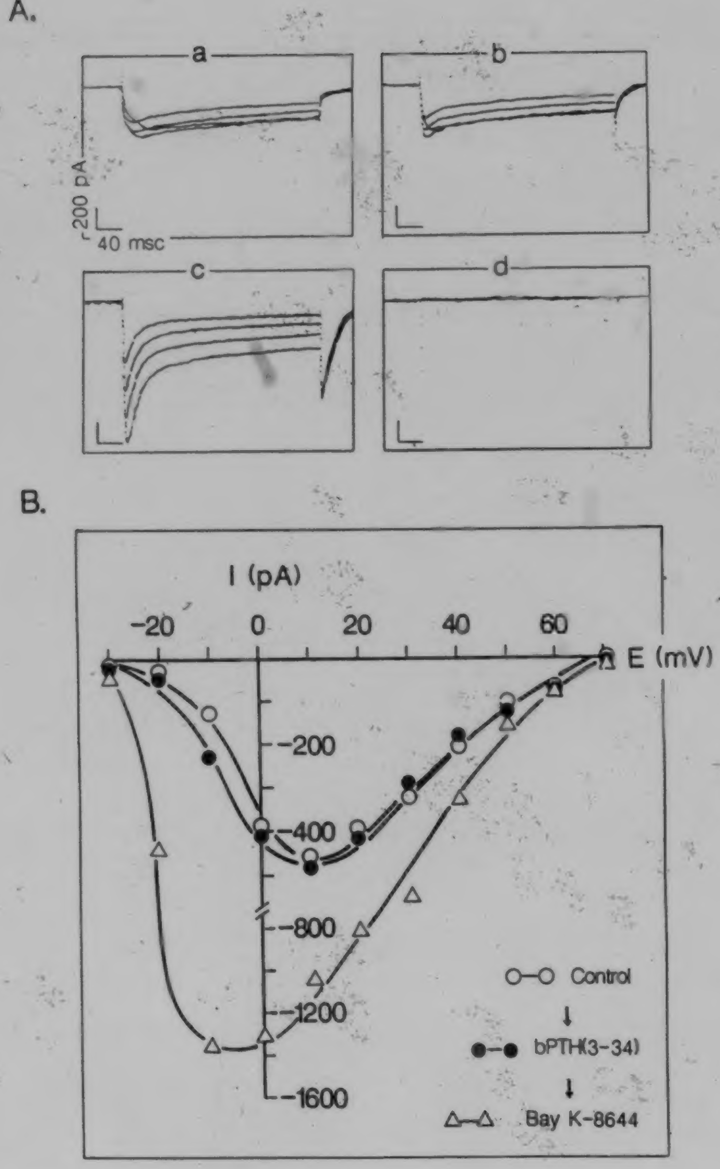

A.
a
b
200 pA
40 msc
c
d
B.
I (pA)
-20
0
20
40
60
E (mV)
-200
-400
-800
-1200
-1600
○–○ Control
↓
●–● bPTH(3-34)
↓
△–△ Bay K-8644

Fig. VIII-8. The effect of 1 μM bPTH-(3-34) on the amplitude of L channel currents in one ventricular myocyte. The same cell was used in both **A** and **B**. Leakage and capacitive currents have been subtracted from original current traces (HP -40 mV). **A.** L channel current traces elicited from 0 to 30 mV in a 10 mV stepwise increment **(a)** under control conditions, **(b)** in the presence of 1 μM bPTH-(3-34), **(c)** after application of 5 μM Bay K-8644 and **(d)** 2 mM La^{+++}, sequentially. **B.** The I-V relationships of L channel currents under various conditions. bPTH-(3-34) did not change the amplitude of L channel currents. Subsequent application of Bay K-8644 increased the amplitude and shifted the peak of the I-V relationship of the L channel current.

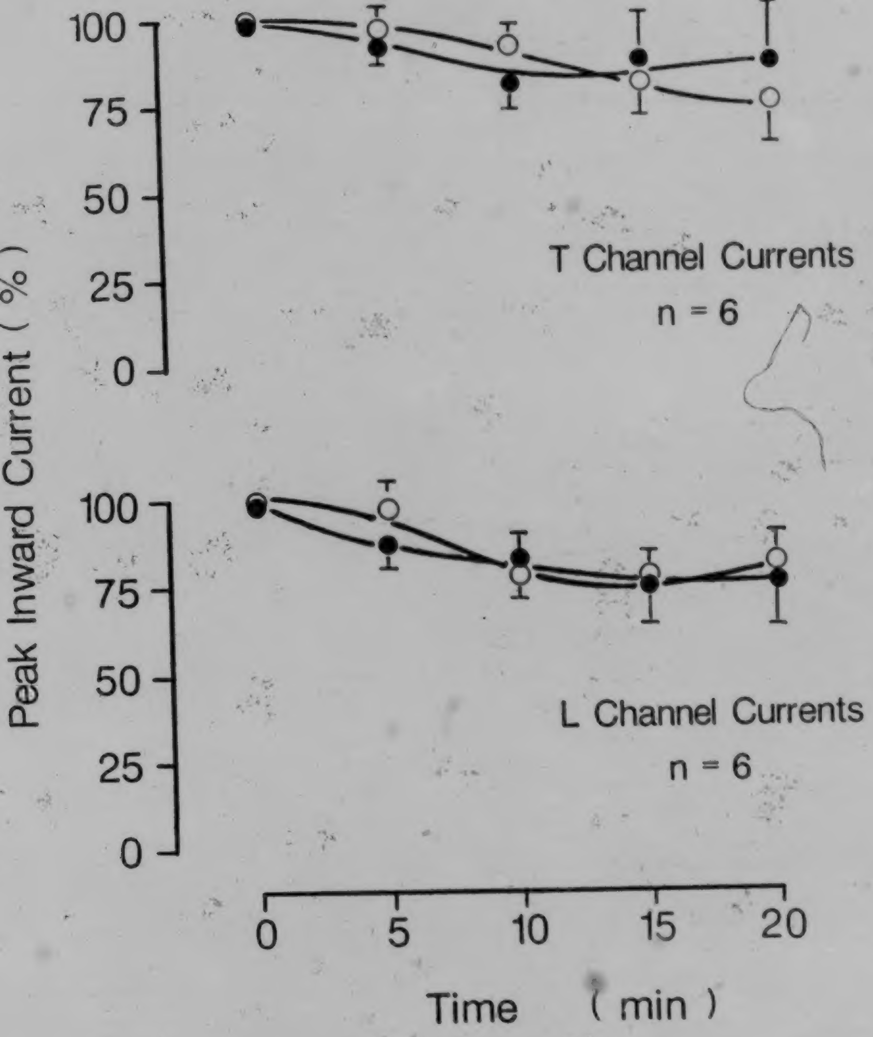

Peak Inward Current (%)
100
75
50
25
0
T Channel Currents
n = 6
100
75
50
25
0
L Channel Currents
n = 6
0
5
10
15
20
Time (min)

Fig. VIII-9. Time-related changes in the amplitudes of T and L channel currents in the absence (open circles) or presence (filled circles) of 1 μM bPTH-(3-34) in ventricular myocytes. There were no significant differences in the amplitudes of T or L channel currents between the two conditions within a time period of 20 min.

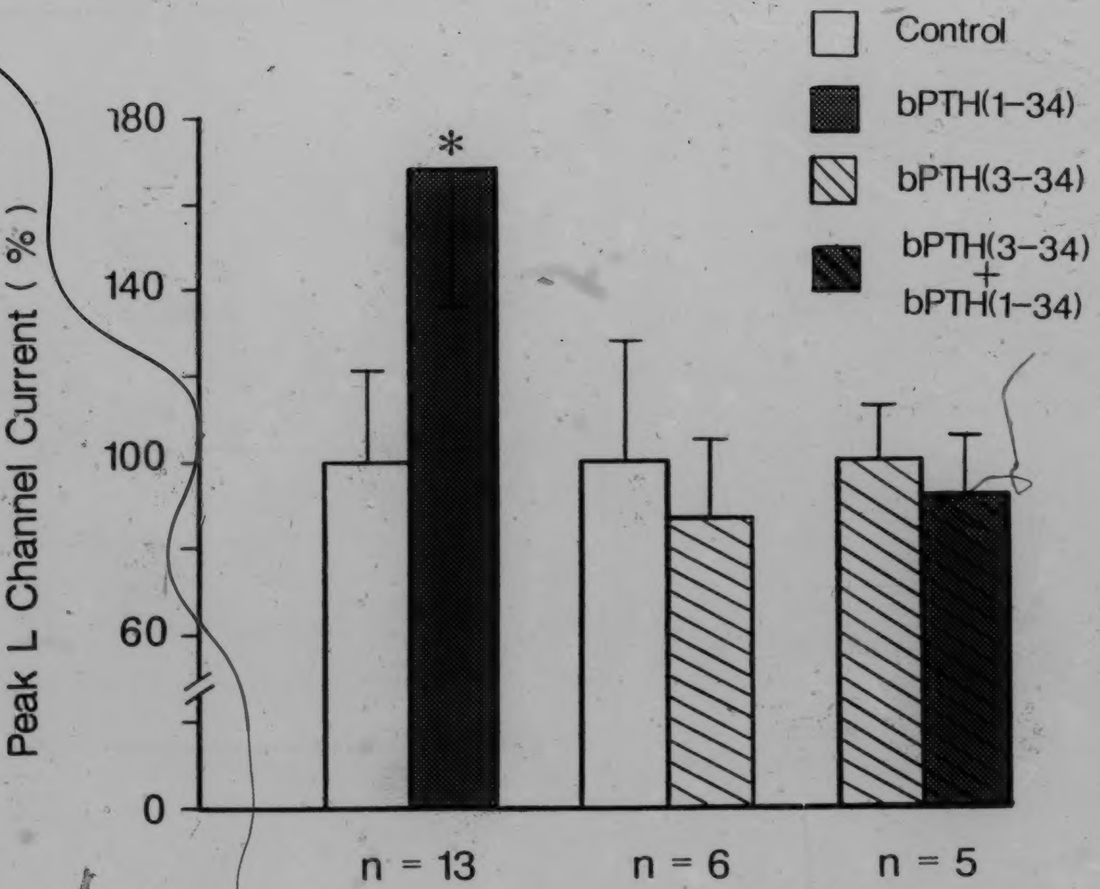

Control
bPTH(1–34)
bPTH(3–34)
bPTH(3–34) + bPTH(1–34)
180
140
100
60
0
Peak L Channel Current (%)
*
n = 13
n = 6
n = 5

Fig. VIII-10. The interaction of the effects of bPTH-(3-34) and bPTH-(1-34) on the amplitudes of L channel currents in ventricular myocytes. bPTH-(1-34) at a concentration of 1 μM increased the amplitude of L channel currents by 73 ±13% ($P<0.05$). However, 1 μM bPTH-(3-34) did not affect the amplitude of L channel currents (87 ±28%, $p>0.05$). After pretreating cells with 10 μM bPTH-(3-34) for more than 15 min, the subsequent application of 1 μM bPTH-(1-34) failed to affect the amplitude of L channel currents.

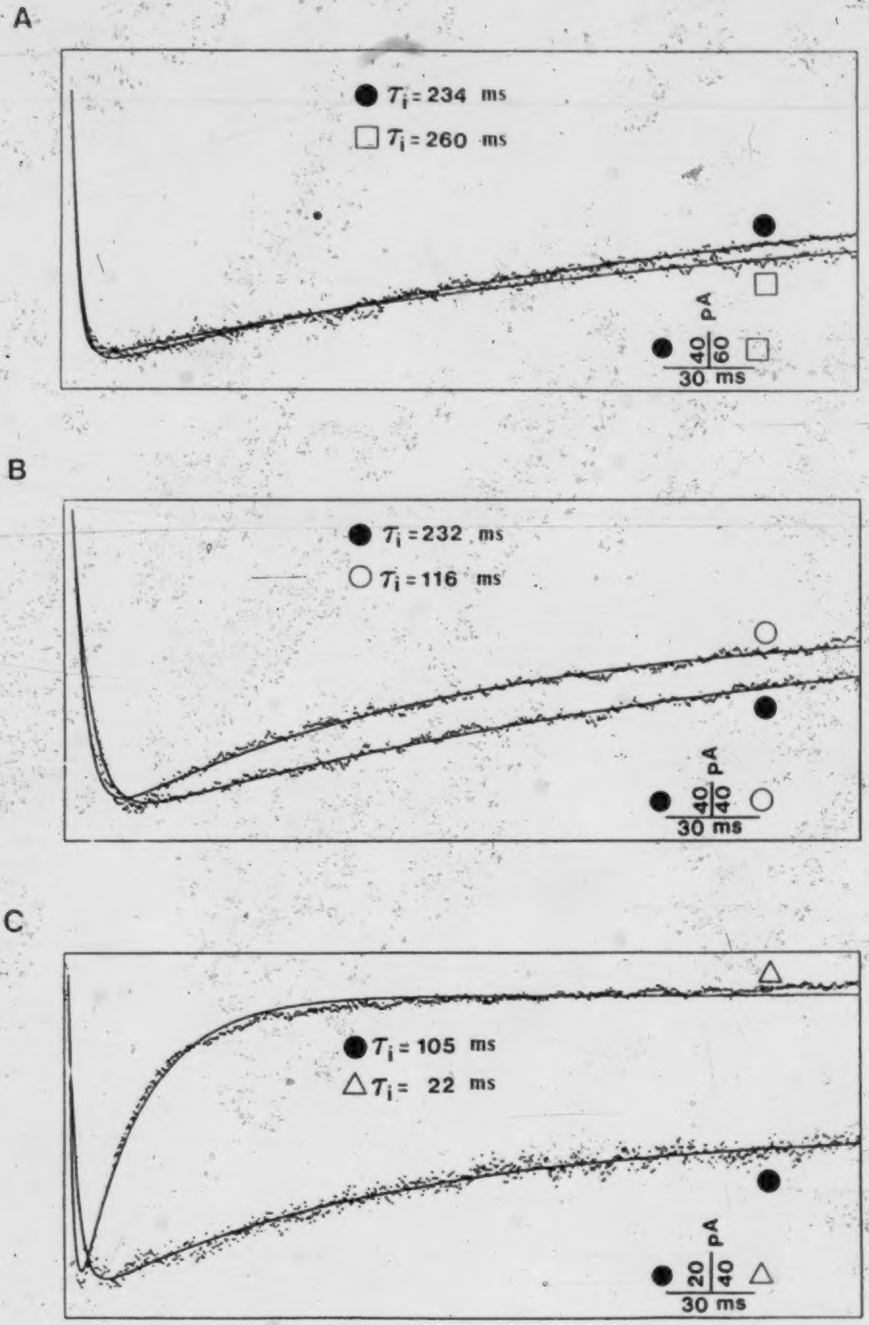

A
● τ_i = 234 ms
□ τ_i = 260 ms
●
□
● 40 | 60 □ pA
30 ms
B
● τ_i = 232 ms
○ τ_i = 116 ms
○
●
● 40 | 40 ○ pA
30 ms
C
△
● τ_i = 105 ms
△ τ_i = 22 ms
●
● 20 | 40 △ pA
30 ms

Fig. VIII-11. Changes in the inactivation kinetics of L channel currents in ventricular myocytes under different experimental conditions. Filled circles indicate the current traces under control conditions. Open squares indicate current traces in the presence of 1 μM bPTH-(1-34) (**A.** Cell #I0518). Open circles indicate current traces in the presence of 1 μM bPTH-(3-34) (**B.** Cell # D0411). Open triangles indicate current traces in the presence of 5 μM Bay K-8644 (**C.** Cell # H0214). The calibration bars for the amplitudes of currents in **A** and **C** represent different values.

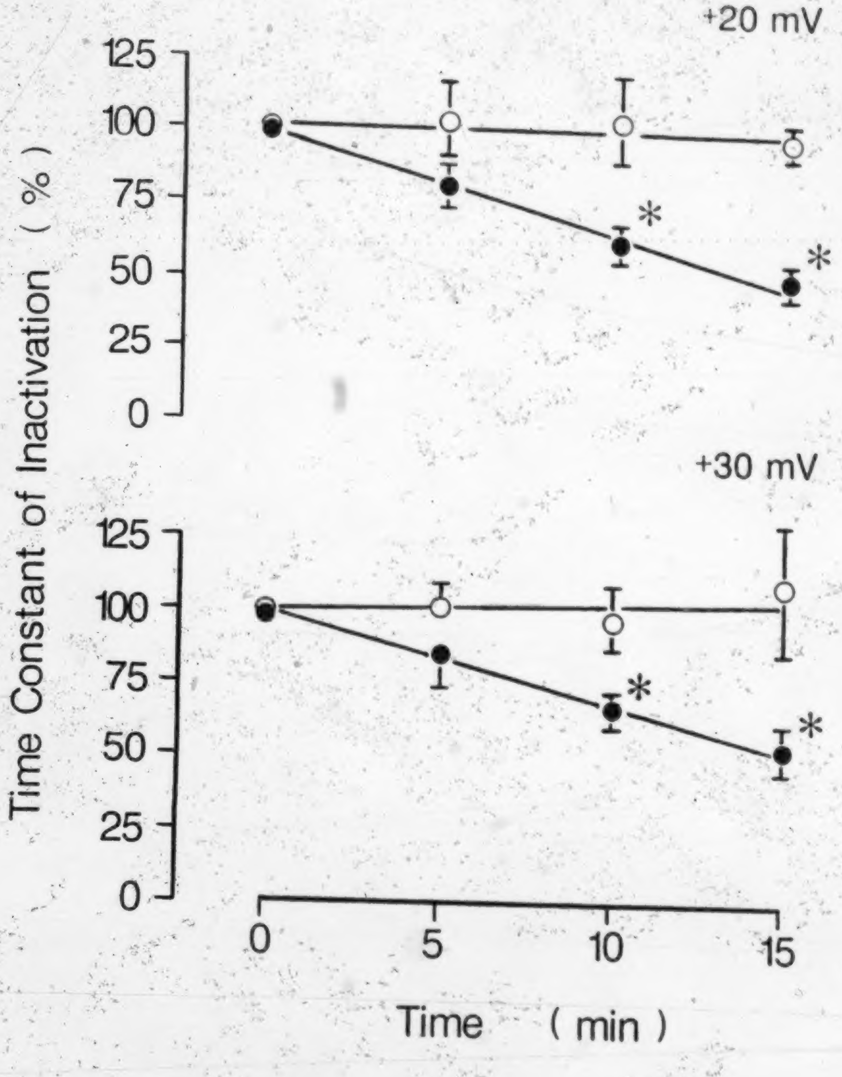

+20 mV
+30 mV
Time Constant of Inactivation (%)
125
100
75
50
25
0
125
100
75
50
25
0
*
*
*
*
0
5
10
15
Time (min)

Fig. VIII-12. Time-related changes in the inactivation kinetics of L channel currents in the absence (open circles) or presence (filled circles) of 1 μM bPTH-(3-34) in ventricular myocytes. A significant change in the inactivation kinetics of L channel currents was detected 10 min after application of bPTH-(3-34) to the bath. * indicates $p < 0.05$. Each point in the figure represent 5 or 6 cells.

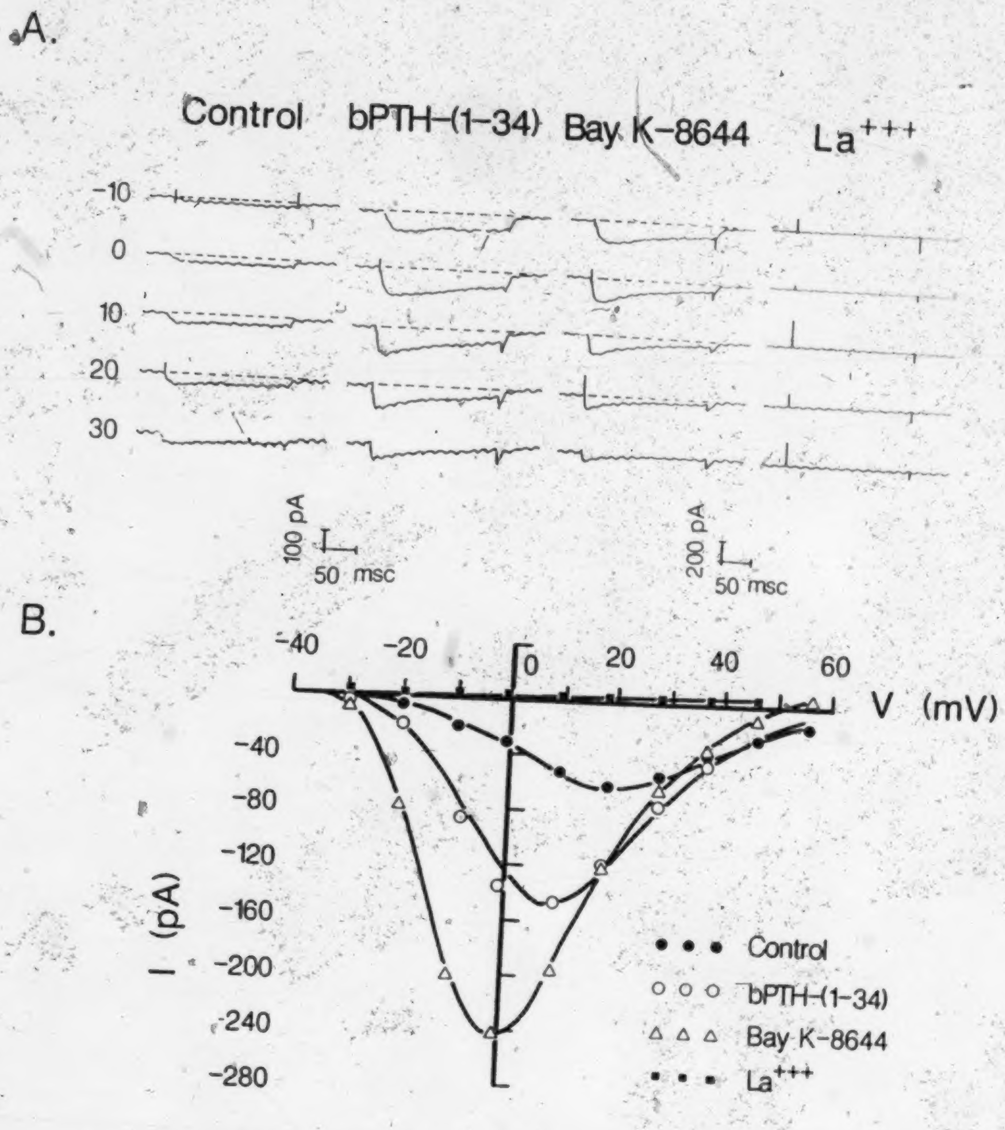

A.
Control
bPTH-(1-34)
Bay K-8644
La+++
-10
0
10
20
30
100 pA
50 msc
200 pA
50 msc
B.
-40
-20
0
20
40
60
V (mV)
-40
-80
-120
-160
-200
-240
-280
I (pA)
● ● ● Control
○ ○ ○ bPTH-(1-34)
△ △ △ Bay K-8644
▪ ▪ ▪ La+++

Fig. VIII-13. The response of L channel currents to bPTH-(1-34) and Bay K-8644 in ventricular myocytes. **A.** Depolarization to different membrane potentials, indicated in mV beside each trace, from a holding potential of -40 mV elicited L channel currents. 1 μM bPTH-(1-34) amplified L channel currents. Subsequent application of 5 μM Bay K-8644 further enhanced the L channel currents. 2 mM La^{+++} removed the inward current completely. Note that the scale of the current intensity for the left two panels is different from that for the right two panels. **B.** The I-V relationships of L channel currents under different conditions. The leakage and capacitive currents have been subtracted in both **A** and **B**.

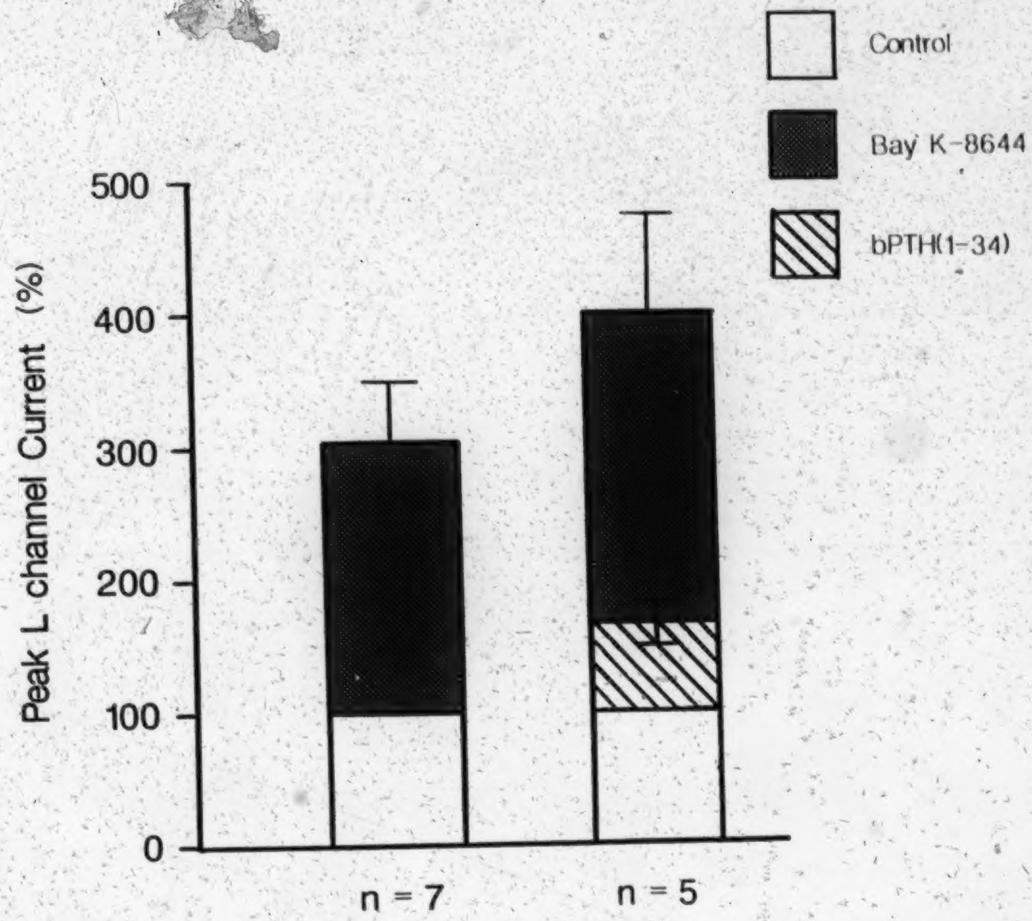

Control
Bay K-8644
bPTH(1-34)
Peak L channel Current (%)
500
400
300
200
100
0
n = 7
n = 5

Fig. VIII-14. The increase in the amplitude of L channel currents in ventricular myocytes by the addition of 5 μM Bay K-8644 in the absence or presence of 1 μM bPTH-(1-34). The amplitude of L channel currents in the absence of any agents was taken as the control (100%). In the absence of bPTH-(1-34), Bay K-8644 increased the L channel currents by 202±38% ($n=7$, $p<0.05$). At 1 μM, bPTH-(1-34) increased the L channel current by 66±20% ($n=5$, $p<0.05$). After the effect of bPTH-(1-34) on L channel currents reached the maximal level, subsequent application of Bay K-8664 increased the L channel currents by an additional 164±51% ($p<0.05$). However, no significant difference in the effect of Bay K-8644 in the absence or presence of bPTH-(1-34) was detected ($p>0.05$).

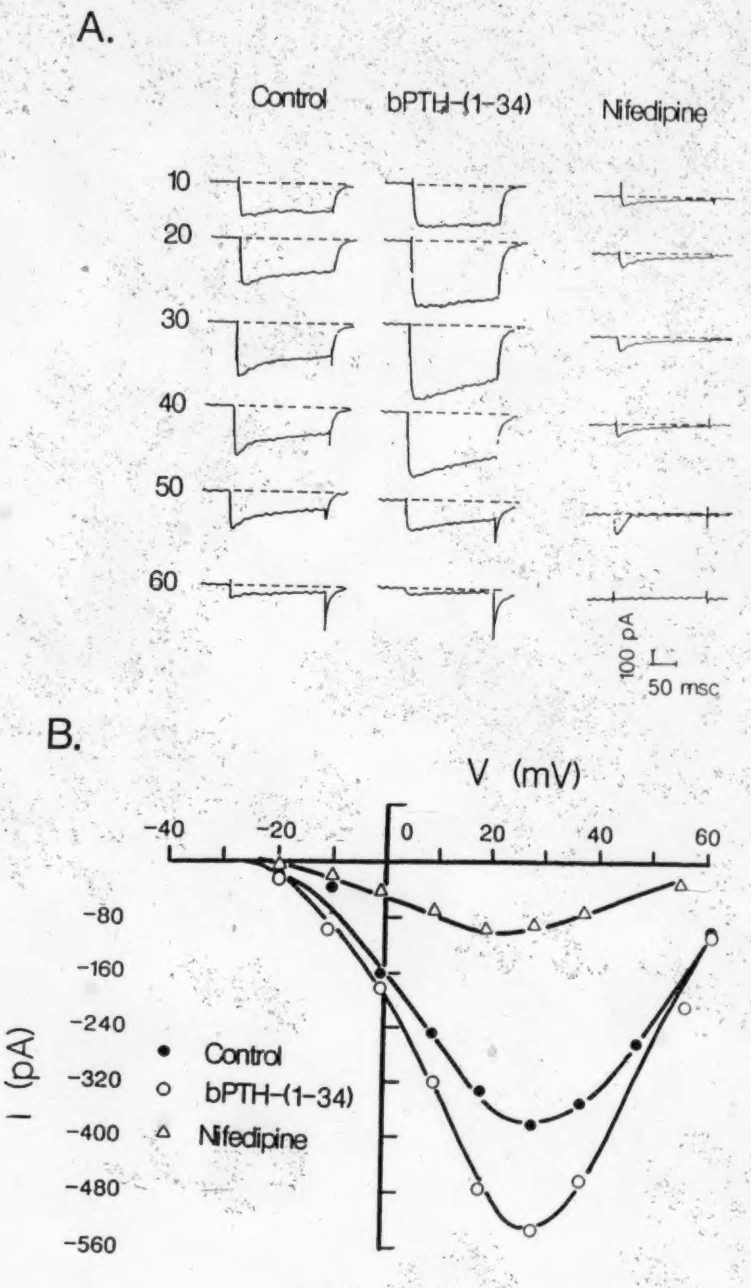

A.
Control
bPTH-(1-34)
Nifedipine
10
20
30
40
50
60
100 pA
50 msc
B.
V (mV)
-40
-20
0
20
40
60
-80
-160
-240
-320
-400
-480
-560
I (pA)
● Control
○ bPTH-(1-34)
△ Nifedipine

Fig. VIII-15. The response of L channel currents in ventricular myocytes to bPTH-(1-34) and nifedipine. **A.** Depolarization to different membrane potentials, indicated in mV beside each trace, from a holding potential of -40 mV elicited L channel currents. In the presence of 1 μM bPTH-(1-34), L channel currents were increased. Subsequent application of 10 μM nifedipine eliminated most of the L channel current. **B.** The I-V relationships of L channel currents under different conditions. The leakage and capacitive currents have been subtracted in both **A** and **B**.

Table VIII-1

Summary of the Kinetic Changes in L Channel Currents in Ventricular Myocytes under Different Experimental Conditions

		Time constant of activation (ms)	n	p	Time constant of inactivation (ms)	n	p
bPTH-(1-34)	Before	1.81 ± 0.17	8	<0.05	160.4 ± 50.3	8	>0.05
	After	2.49 ± 0.29			181.5 ± 43.5		
bPTH-(3-34)	Before	2.01 ± 0.54	6	>0.05	158.9 ± 51.7	6	<0.05
	After	1.83 ± 0.36			117.4 ± 42.7		
(3-34) + (1-34)	Before	2.14 ± 0.45	5	>0.05	194.4 ± 46.2	5	>0.05
	After	2.10 ± 0.38			162.2 ± 59.8		
Bay K-8644	Before	2.24 ± 0.35	6	<0.05	142.0 ± 24.6	6	<0.05
	After	1.01 ± 0.13			24.2 ± 3.1		

CHAPTER 9

The morphological changes in single vascular smooth muscle cells and ventricular myocytes induced by bPTH-(1-34)

INTRODUCTION

In vitro studies have established the vasodilatory and cardiotonic effects of PTH peptide. However, the direct effect of PTH on the relaxation of vascular smooth muscle cells and the contraction of ventricular myocytes at the single cell level have not been demonstrated. The following question, therefore, is asked: is the relaxation of vascular tissue induced by PTH mediated by another unknown transmitter or by a local neuronal reflex, rather than through a direct effect of PTH on vascular smooth muscle cells. It has been demonstrated that bPTH-(1-34) inhibited L-type voltage-dependent calcium channels in single neuroblastoma cells and vascular smooth muscle cells. Furthermore, bPTH-(1-34) increased the amplitude and changed the rate of the activation of L channel currents in single neonatal rat ventricular cells. However, it is not clear whether the effect of PTH on voltage-dependent calcium channel currents in these cell preparations could be related to the functional changes induced by PTH.

The purpose of the present study is to provide an indication of whether the changes in Ca^{++} channel currents in the presence of bPTH-(1-34) can be correlated with the functional response of the primary cultured vascular smooth muscle cells and ventricular myocytes under the present experimental conditions. Hence, the influence of bPTH-(1-34) on the shape or beating rate in cultured cells was studied.

RESULTS

The effect of bPTH-(1-34) on the contraction of smooth muscle cells

The areas of smooth muscle cells in culture medium at room temperature were 403 ± 22 square micra (n=13). When the cells were perfused with the modified Tyrode solution containing 60 mM KCl, the area of the cells decreased gradually to a minimum area (78.5 ± 4% of the control value) occurring at 5 min. Thereafter, the cells started to recover from the contraction even though the difference in the cell area at 5 min and 10 min after KCl challenge was not significant ($P > 0.05$).

In the second set of experiments, the cells were superfused with modified Tyrode solution containing 60 mM KCl and 1 μM bPTH-(1-34). When measured within the first minute of perfusion, the cell areas were 95 ± 3% of the control (n

= 18), which was not significantly different from cells exposed to the modified Tyrode solution with 60 mM KCl but without bPTH-(1-34) ($p > 0.05$). However, the cell areas increased continuously during the ensuing time (Fig. IX-1). The difference in the cell areas between the cells exposed to the modified Tyrode solutions with and without bPTH-(1-34) was significant ($p<0.05$).

The effect of bPTH-(1-34) on the contraction of ventricular myocytes

The areas of quiescent ventricular myocytes in the culture media at room temperature were 516 ± 47 square micra (n = 29), which is comparable to the size of embryonic chick ventricular myocytes (Wahler *et al.*, 1990). The myocytes contracted in the presence of 1 μM bPTH-(1-34) in a time-dependent manner. After bPTH-(1-34) was added to the bath solution, the area of myocytes decreased gradually within 10 min. However, no significant difference in the cell area could be found during this period of time. The relative cell areas were 96 ± 2% and 94 ± 2% at 5 min and 10 min, respectively, after addition of bPTH-(1-34) ($P>0.05$). At 15 min, the cell area decreased significantly to 88 ± 2%, taking the control area of the cells as 100% ($n=9$, $p<0.05$). To study the possibility that the effect of PTH on cell contraction was related to the modulation of voltage-dependent calcium channels in these myocytes, nifedipine, a classical L-type voltage-dependent calcium channel antagonist, was subsequently applied to the cells. As shown in Fig. IX-2, nifedipine at a final concentration of 1 μM completely inhibited the contraction of myocytes elicited by 1 μM bPTH-(1-34). This result further indicated that the effect of bPTH-(1-34) on the contraction of myocytes was due, at least in part, to the activation of calcium channels.

The effect of bPTH-(1-34) on the beating rate of ventricular myocytes

After culture for more than 24 hrs, most of the myocytes maintained slow but stable beating rhythms at room temperature. In several cells, the chronotropic effect of bPTH-(1-34) was immediately seen. However, in most cases, the effect of bPTH-(1-34) on the beating rate became significant only 5 min after application. Thereafter, the beating rate stabilized at the increased level for the observation time of 20 min. In 7 cells, bPTH-(1-34) increased the beating rate by 145 ± 45%. In another three cells, no spontaneous beating could be detected at the beginning. After the application of bPTH-(1-34), the contractile frequency was 58 ± 9 min^{-1}. The chronotropic effect of bPTH-(1-34) was also related to the influx of Ca^{++} through voltage-dependent calcium channels. Evidence for this came from the effect of 1 μM nifedipine on the beating rate. When nifedipine was added to the bath solution in the presence of bPTH-(1-34), the increased beating rate elicited by bPTH-(1-34) was decreased within 1 min to the basal rate and declined continuously until it stopped completely during the ensuing 5 min (Fig. IX-3). This result also indicated that, under the present experimental conditions, the opening of the calcium channels was possibly responsible for the basal spontaneous firing of the action potential in these myocytes.

DISCUSSION

bPTH-(1-34) has been reported by two different laboratories to affect the cAMP content in isolated smooth muscle cells directly (Nickols, 1985; Stanton *et al.*, 1985). However, evidence indicating that PTH can directly relax isolated single vascular smooth muscle cells is not available at the present time (Mok *et al.*, 1989a). In the present study, KCl was used to induce smooth muscle cell contraction and then bPTH-(1-34)-induced cell relaxation was observed. KCl induced smooth muscle contraction is composed of two phases. A rapid contraction, the phasic phase, was followed by a prolonged contraction plateau, the tonic phase (Hurwitz *et al.*, 1980; Villar *et al.*, 1986). Different mechanisms are proposed to explain these two phases of contraction. Extracellular application of a high concentration of KCl depolarizes the surface membrane and hence opens L-type voltage-dependent calcium channels. If the entry of extracellular calcium sufficiently increases intracellular free calcium concentration ($[Ca^{++}]_i$), smooth muscle contraction occurs. The excitation-contraction latency is about 200 ms (Marshall, 1974). The contraction lasts for more than one hour. This prolonged contraction in the mesenteric artery strip was inhibited by dihydropyridine antagonists (Asano *et al.*,1988). Extracellular application of verapamil, lanthanum ion and EGTA also inhibited the tonic contraction of guinea-pig ileum smooth muscle (Hurwitz *et al.*,1980). It seems that voltage-dependent calcium channels are critical for both phasic and tonic contraction of smooth muscle cells induced by KCl (Brading *et al.*, 1983; Huddart and Butler, 1983; Langton and Huddart, 1987). Within seconds L channels will, however, inactivate owing to both increased $[Ca^{++}]_i$ and membrane depolarization (Ohya *et al.*, 1988; Pelzer *et al.*, 1990). The difference in the experimental conditions in tension studies and patch clamp recording may help us to understand this puzzling and unsolved discrepancy. It should be pointed out that the mechanism of KCl-induced smooth muscle contraction and the role played by voltage-dependent calcium channels are still far from clear (Urakawa and Holland, 1964). The source for increased $[Ca^{++}]_i$ during maintained depolarization and contraction may also be calcium-induced calcium release (CICR) from sarcoplasmic reticulum (SR) (Van Breemen and Saida, 1989). If plasmalemma calcium channels are inhibited, less entry of extracellular calcium during depolarization will possibly lead to less CICR. The outcome will be the inhibition of both tonic and phasic contraction. However, the calcium content of SR in vascular smooth muscle cells is limited. Without extracelluar calcium the smooth muscle contraction during sustained KCl-elicited depolarization cannot be maintained for hours. In chicken gizzard smooth muscle, high K^+ only induced a single phasic phase which was completely dependent on extracellular calcium (Ozaki *et al.*, 1990). No CICR could be demonstrated in this smooth muscle preparation. Another argument against the importance of CICR in smooth muscle cells was derived from the reversibility of KCl effects on both smooth muscle contraction and $[Ca^{++}]_i$. After removal of 90 mM KCl from the organ bath, the porcine coronary artery immediately relaxed and $[Ca^{++}]_i$ returned to the pre-contraction level (Mori *et al.*, 1990). If the tonic contraction is due to CICR, the presence of KCl should not be essential once the plasmalemmal calcium channels fully opened. In light of these considerations, the extracellular calcium entry may be the determinant and a more important factor than CICR in KCl-induced vascular smooth muscle contraction (Karaki, 1987; Rembold, 1989). PTH effects on single smooth muscle cell contraction induced by 60 mM KCl may be explained by the above mechanism. In the present study, the composition of bath solution is closer to extracellular solution *in vivo* than that used in the patch clamp studies. The native cytosol environment was not disturbed as it was during dialysis in the patch clamp studies. bPTH-(1-34) started to inhibit cell contraction initiated by KCl even at the first minute ($p > 0.05$). Within the ensuing 5 min, smooth muscle cells were completely relaxed in the presence of bPTH-(1-34). These results are consistent with tension studies in which bPTH-(1-34) inhibited the tonic contraction of rat tail artery strips induced by 60 mM KCl (Pang et al., 1988a). A simple but reasonable explanation for the present results is that bPTH-(1-34) may relax single vascular smooth muscle cells by inhibiting plasmalemma calcium channels, and/or, indirectly, CICR. However, this interpretation of PTH effects by no mean is the only or entire mechanism. For example, PTH increased cAMP concentration in vascular smooth muscle cells in a time-dependent fashion (Mok, *et al.*, 1989a). Not only L channel currents (see Chapter 10) but also the sensitivity of myosin light chain kinase to calcium will be decreased (Adelstein *et al.*, 1978; Conti and Adelstein, 1980; Taylor *et al.*, 1989). PTH may also directly affect CICR (Filburn and Harrison, 1990), modulate other ionic channels on the surface membrane, and alter the activities of the Na/Ca exchanger (Fraser et al., 1988a, b) and calcium pump (Mckenzie, et al., 1990). Nevertheless, the results from the present study demonstrated that bPTH-(1-34) can directly inhibit KCl-elicited contraction of single vascular smooth muscle cells in a time-dependent manner in which the modulation of L channels by PTH may be important.

The effects of PTH on cardiac tissues were mainly demonstrated with *in vitro* heart perfusion preparations (Hashimoto *et al.*, 1981; Nickols *et al.*, 1989a), isolated papillary muscle (Katoh *et al.*, 1981), sinus node cells (Kondo *et al.*, 1988) and rat right atria (Tenner *et al.*, 1982). Bogin *et al.* (1981) investigated the effect of bPTH-(1-34) on the beating rate and cAMP content of cultured heart cells from newborn rats. However, these myocardial cells were taken from the whole heart. When the contractile frequency was recorded, it was difficult to separate the response of atrial cells to PTH from that of ventricular cells. To date, the direct effects of PTH peptides on the function of single isolated ventricular cells, either on contraction or beating, have not been reported. If single ventricular myocytes were first exposed to a high concentration of KCl, the cell would contract to such a degree that the additional contraction induced by bPTH-(1-34) could not be easily detected. Therefore, in the present study, 1 μM bPTH-(1-34) was directly applied to ventricular myocytes in the absence of KCl. Under the same enzymatic dispersion and culture conditions but with different compositions of extra- and intracellular solutions from those used in the patch clamp studies (Chapter 8) on ventricular cells, bPTH-(1-34) elicited contraction and increased the beating rate of ventricular myocytes. Concerning this experimental design, the question arose as to whether bPTH-(1-34)-induced contraction was due to the modulation of voltage-dependent calcium channels on surface membrane. As shown in Chapter 8 (Fig. VIII-5), bPTH-(1-34) shifted the normalized conductance curve of L channels to the direction of more negative potentials. Therefore, L channels in ventricular myocytes will be able to open without a significant change of the resting potential in the presence of bPTH-(1-34) (Casteels *et al.*, 1990). In addition, the effect of bPTH-(1-34) on the function of single myocytes could be abolished by nifedipine, an antagonist of L-type voltage-dependent calcium channels. These results suggested that bPTH-(1-34) alone might increase voltage dependent extracellular calcium entry in single ventricular myocytes. The increased influx of calcium alone or together with CICR, which may be more important than in vascular smooth muscle, produced ventricular myocyte contraction. Again, PTH-induced ventricular myocyte contraction may have multiple mechanisms. A clear picture of how PTH alone induces cell contraction can not be drawn without further investigation. The result that bPTH-(1-34) contracted single ventricular myocytes is in line with the bPTH-(1-34)- induced increase in L channel currents (Chapter 8) and, as such, provides a functional basis for the electrophysiological data.

The isolated primary cultured heart cells represent normally functioning myocytes of the intact heart (Bogin *et al.*, 1981). This statement is substantiated by electrophysiological evidence (Powel *et al.*, 1980; Isenberg and Klöckner, 1982), biochemical studies (Geisbuhler *et al.*, 1984; Bihler *et al.*, 1985), and morphological observations (Piper *et al.*, 1982; Severs *et al.*, 1982; Slade *et al.*, 1983). Data presented in this chapter, together with other reports in the literature, indicate that the cardiotonic effects of PTH can be observed under *in vivo* or *in vitro* experimental conditions, either at the single cell level or at the tissue level.

It is always difficult to deduce physiological significance from results of patch clamp studies. Using the patch clamp technique to study voltage-dependent calcium channels, the influence of other ionic channels, such as sodium, potassium and chloride channels must be excluded. Therefore, cells are bathed with sodium-free extracellular solution and dialysed with potassium-free intracellular solution. In order to facilitate the recording of the small inward calcium current, a high concentration of barium is used. Intracellular free calcium is chelated with EGTA to rule out the possible calcium-induced inactivation of calcium channels. Without these unusual conditions, the observation of calcium channel changes is impossible.

However, these non-physiological conditions make it difficult to interpret the functional significance of these patch clamp results. In this regard, the correlation of patch clamp studies with functional studies under physiological conditions is exceptionally important. This aim is partially achieved with the morphological studies presented here. These results show that the same vascular smooth muscle and ventricular muscle cells under conditions more closely resembling physiological circumstances have functional responses to bPTH-(1-34) which can be correlated with the bPTH-(1-34) effect on L channel currents under patch clamp conditions.

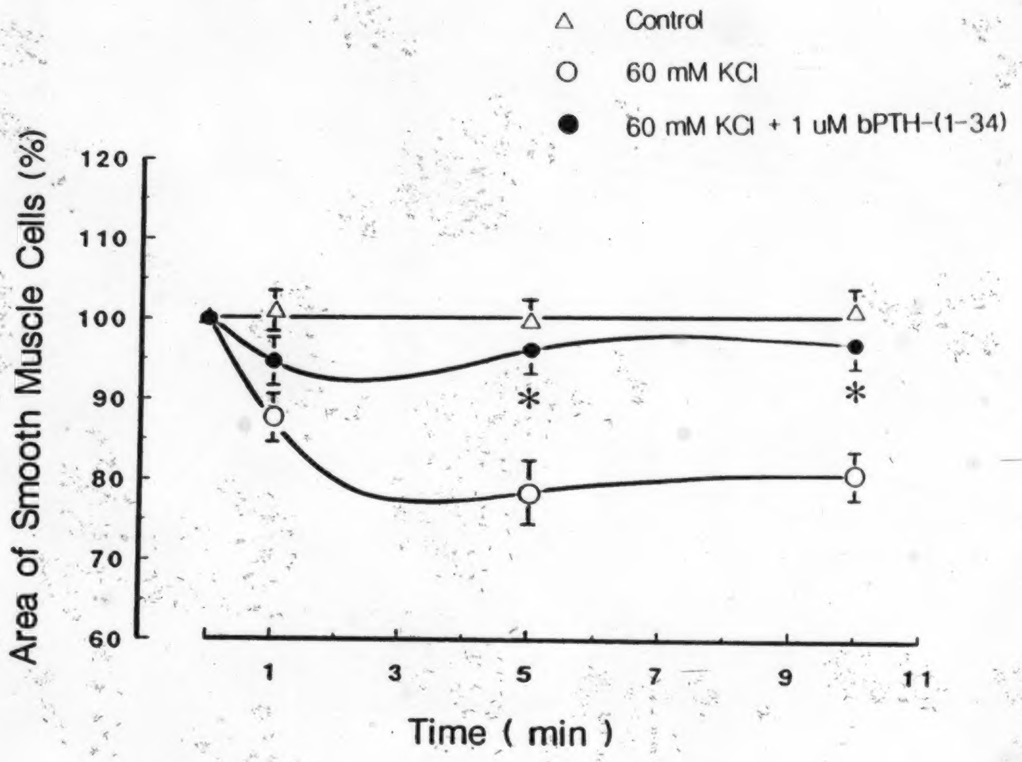

△ Control
○ 60 mM KCl
● 60 mM KCl + 1 uM bPTH-(1-34)
Area of Smooth Muscle Cells (%)
120
110
100
90
80
70
60
*
*
1
3
5
7
9
11
Time (min)

Fig. IX-1. The effect of bPTH-(1-34) on the contraction of single vascular smooth muscle cells induced by KCl challenge. bPTH-(1-34) at 1 μM inhibited KCl-elicited contraction of smooth muscle cells in a time-dependent manner. The number of cells tested at each time point ranged from 13 to 18. * indicates $p < 0.05$ when comparison is made before and after the application of bPTH-(1-34) in the presence of KCl.

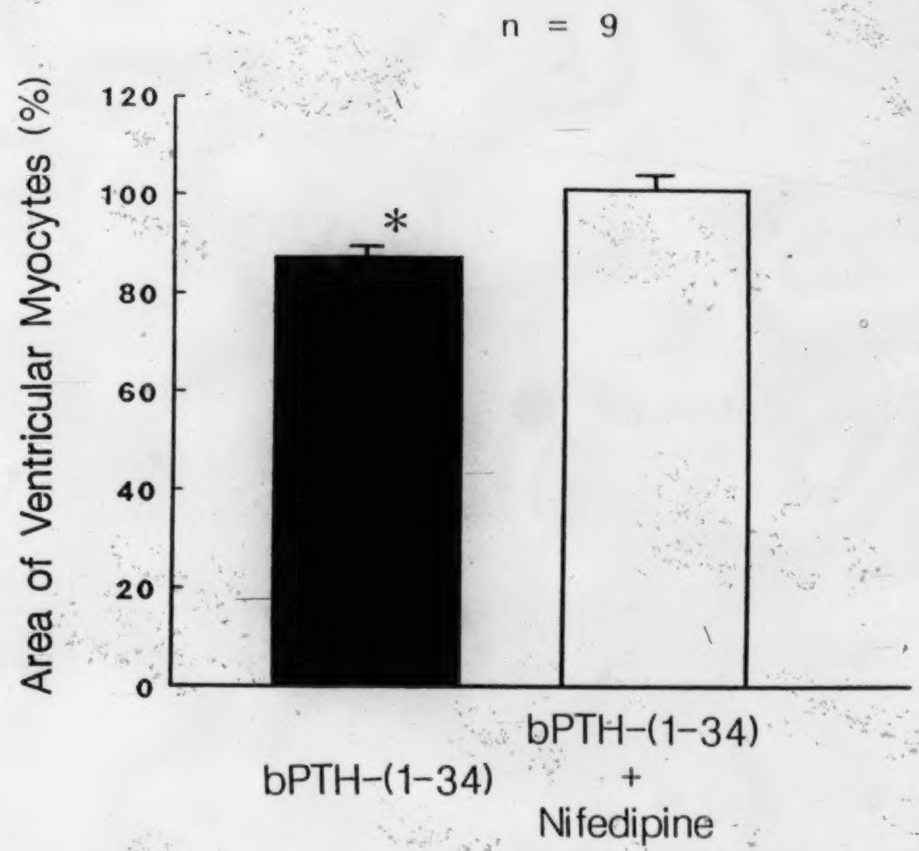

n = 9
Area of Ventricular Myocytes (%)
120
100
80
60
40
20
0
*
bPTH-(1-34)
bPTH-(1-34)
+
Nifedipine

Fig. IX-2. The effect of bPTH-(1-34) on the contraction of single ventricular myocytes. bPTH-(1-34) at 1 μM significantly deceased the area of ventricular myocytes. This effect of bPTH-(1-34) was antagonized by nifedipine at a concentration of 1 μM. * indicates $p < 0.05$.

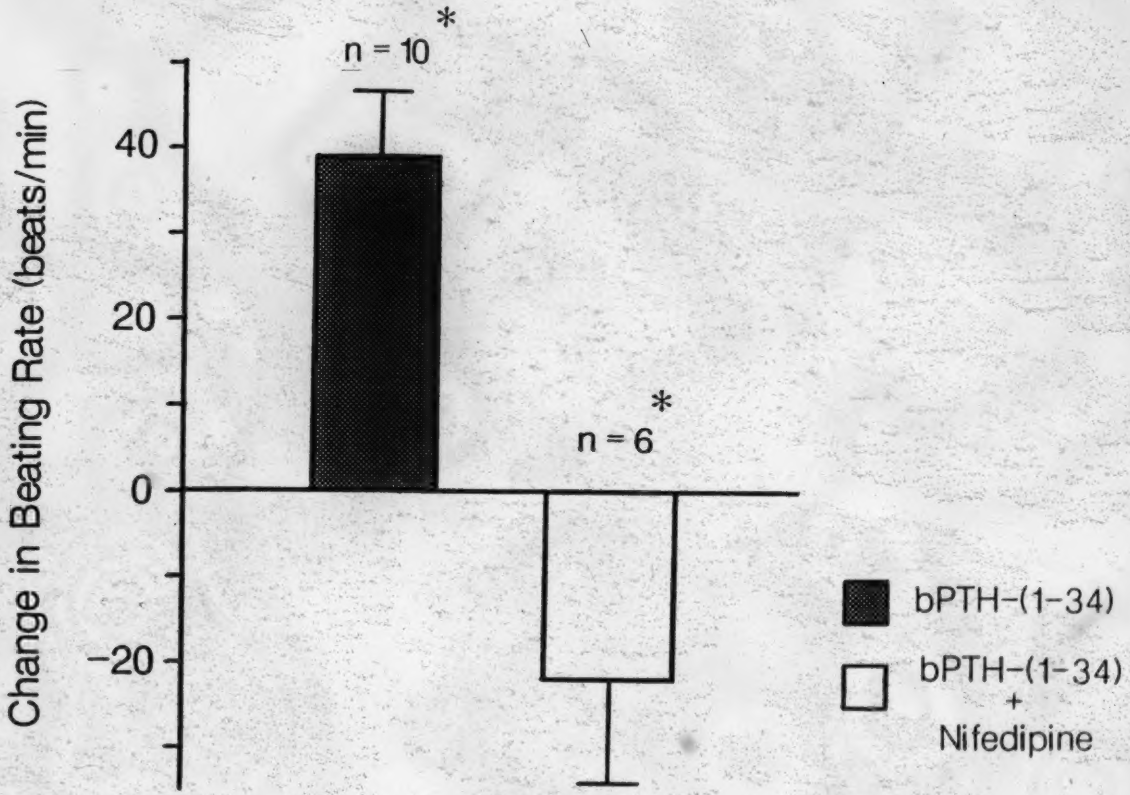

Change in Beating Rate (beats/min)
40
20
0
−20
n = 10
*
n = 6
*
bPTH-(1-34)
bPTH-(1-34)
+
Nifedipine

Fig. IX-3. The change in beating rate of single ventricular myocytes induced by bPTH-(1-34) and nifedipine. At 1 μM, bPTH-(1-34) significantly increased the beating rate of ventricular myocytes. However, nifedipine at 1 μM significantly decreased the PTH-induced increase in the beating rate. * indicates $p < 0.05$.

CHAPTER 10

Cyclic AMP mediation of bPTH-(1-34) effects on L channel currents in vascular smooth muscle cells and ventricular myocytes

INTRODUCTION

PTH increases intracellular cAMP content in cells of its target tissues, including renal cortical membranes (Goltzman *et al.*, 1976), tumor and normal osteoblast-like cells (Helwig *et al.*, 1984; Rodan and Rodan, 1984; Ferrier *et al.*, 1988), vascular smooth muscle (Pang *et al.*, 1986; Nickols and Cline, 1987) and right atrium (Tenner *et al.*, 1983).

Production of cAMP also plays an important role in the vascular effect of PTH (Musso *et al.*, 1989). In isolated vascular smooth muscle cells, the increase in cAMP content occurred within 1 min and was maximal within 5 to 10 min after treatment with bovine (b) PTH-(1-34) (Nickols 1985). Using the same tissue, it was reported that the relaxation of vessel strips induced by bPTH-(1-34) was immediate but the maximum effect of PTH did not develop until 6 min. In agreement with this, a period of 5 min was also needed to establish the inhibitory effect of bPTH-

(1-34) on L channel currents from rat tail artery smooth muscle cells in our patch clamp studies. The similarity in the temporal sequence of PTH effects on cAMP content, calcium channel currents and relaxation of vascular tissues is consistent with the hypothesis that the PTH effect may be mediated at least in part by the cAMP pathway.

Both the biological activity and cAMP promoting effect of PTH could be antagonised by PTH antagonists, such as bPTH-(3-34) and bPTH-(7-34) (see review by Mok *et al.*, 1989a). Oxidized bPTH-(1-34) lost its vasodilatory action as well as its cAMP-stimulating effect in rat tail artery tissues (Pang *et al.*, 1986). According to these observations, it is, therefore, hypothesized that the PTH effects were mediated by the second messenger cAMP in vascular smooth muscle cells.

The increase in intracellular cAMP concentration has been demonstrated to enhance calcium channel currents in myocardial cells (Ono *et al.*, 1989). In cardiac tissue, PTH also enhanced cAMP content prior to its chronotropic action (Tenner *et al.*, 1983).

PTH inhibits L channel currents in vascular smooth muscle cells while it increases L channel currents in ventricular muscle cells. However, PTH increases cAMP content in both of these cell preparations. The question raised then is whether the effect of PTH on L channel currents in these two cell preparations is mediated by cAMP. Direct electrophysiological evidence derived from the present study indicates that cAMP is involved in the modulation of L channel currents by

PTH in both vascular smooth muscle cells and ventricular muscle cells.

RESULTS

Section I: Studies with vascular smooth muscle cells

In the first series of experiments, the effect of cAMP alone on the L channel currents was studied. db-cAMP was added to the bath solution to achieve a final concentration of 1 mM. L channel currents decreased in amplitude and were maintained at this decreased level 5 min after application of db-cAMP. The kinetics and I-V relationship of L channel currents were not changed in the presence of db-cAMP (Fig. X-1). In 9 cells, db-cAMP at a concentration of 1 mM induced a 26% decrease in L channel current amplitude ($p<0.05$).

In the second series of experiments, the summation of the effects of db-cAMP and bPTH-(1-34) was investigated. db-cAMP was first added to the bath solution and the decrease in L channel current was recorded. 1 μM bPTH-(1-34) was then applied to the same cell and the amplitude of L channel currents decreased further (Fig. X-2). After 24.6% inhibition by 1 mM db-cAMP, bPTH-(1-34) induced an additional 12% inhibition in L channel currents. The total inhibition of L channel currents induced by db-cAMP and bPTH-(1-34) was 36.8% ($n=5$) which was similar to the value in the presence of 1 μM bPTH-(1-34) alone. Since the effect of bPTH-(1-34) on L channel currents was almost saturated at a concentration of 1 μM, this result suggested that the effects of db-cAMP and bPTH-(1-34) were additive but not synergistic.

In the third series of experiments, 100 μM Rp-cAMPs, a cAMP analogue, was included in the pipette solution to dialyse the cell. The inhibitory effect of bPTH-(1-34) on L channel currents was abolished with the intracellular infusion of Rp-cAMPs (Fig. X-3). It is interesting that after the intracellular infusion of Rp-cAMPs, the amplitude of L channel currents increased by a small but not significant amount in response to bPTH-(1-34) ($n=5, p>0.05$). To rule out the possibility that Rp-cAMPs directly "desensitizes" the L channel or closely associated proteins so that no response of L channels to agonists or antagonists could be elicited, the effect of Bay K-8644 in the presence of Rp-cAMPs was studied. As shown in Fig. X-4, 5 μM Bay K-8644 increased the amplitude of L channel currents by a factor of two ($n=4, p<0.05$). This result indicates two things. First, Rp-cAMPs did not act directly on L channel proteins. Second, the effect of Bay K-8644 on L channel currents was not mediated by the cAMP pathway.

In addition, the effect of imidazole, a phosphodiesterase stimulator (Pang *et al.*, 1986), was examined. The smooth muscle cells were incubated for more than 20 min in the presence of 1 mM imidazole, and then exposed to bPTH-(1-34). The amplitude of L channel currents were -79 ± 8 pA after pretreatment with imidazole and -73 ± 6 pA after the sequential application of 1 μM bPTH-(1-34). No significant difference could be determined under these two conditions ($n=9$,

$p > 0.05$).

Section II: Studies with ventricular myocytes

Parallel with studies on vascular smooth muscle cells, three series of experiments were carried out using ventricular myocytes.

1). db-cAMP increased L channel currents in myocytes. The peak amplitudes of L channel currents were -365 ± 87 and -428 ± 92 pA before and after the application of db-cAMP ($n=7$, $p<0.05$). The increase in the amplitude of L channel currents emerged at approximately 10 min when the cells were exposed to 1 mM db-cAMP. This level was maintained afterwards. As can be observed in Fig. X-5, the kinetics of the activation of L channels decreased, which was similar to the effect produced by bPTH-(1-34) (see Chapter 8). Furthermore, the peak of the I-V relationship of L channel currents was shifted toward the direction of hyperpolarization by 10 mV. This was also similar to the effect of bPTH-(1-34) (see Chapter 8). The negative shift of the peak of the I-V relationship of L channel currents by db-cAMP was detected in another 4 cells.

2). The increase in L channel currents in the presence of 1 mM db-cAMP was further enhanced by the application of 1 μM bPTH-(1-34). 1 mM db-cAMP increased L channel currents by 17 ± 4%. Subsequent application of bPTH-(1-34) at the maximal effective concentration of 1 μM only increased L channel currents by an additional 51% ($n=5$, $p<0.05$) (Fig. X-6). As reported previously, 1 μM bPTH-(1-34) alone increased L channel currents by 73 ± 13%.

3). The intracellular infusion of 100 μM Rp-cAMPs prior to the application of bPTH-(1-34) totally abolished the excitatory response associated with this peptide. Fig. X-7A shows the effects of 1 μM bPTH-(1-34) in a control cell without Rp-cAMPs in the pipette (left) and in a cell previously dialysed with Rp-cAMPs (right). In the presence of Rp-cAMPs the excitatory effect of bPTH-(1-34), as shown in Fig. X-7A-a, was completely abolished (Fig. X-7A-b). Although the amplitude of L channel currents decreased by 14% in the presence of 1 μM bPTH-(1-34) with Rp-cAMPs infusion, the difference was not significant ($n=7$, $p>0.05$) (Fig. X-7B). In addition, the kinetics of inactivation of L channel currents was changed by bPTH-(1-34) with Rp-cAMPs infusion (Fig. X-7A-a). This could not be observed without Rp-cAMPs in the pipette (Fig. X-7A-a).

Intracellular infusion of Rp-cAMPs failed to inhibit the response of L channel currents to Bay K-8644. As shown in Fig. X-8, Bay K-8644 at 5 μM increased L channel currents by a factor of 4 ($n=5$, $p<0.05$).

DISCUSSION

1. PTH effect was mediated by cAMP in two types of cells. The evidence to support this statement is listed below.

1). In smooth muscle cells, bPTH-(1-34) decreased the amplitude of L channel currents but had no effect on the I-V relationship and the kinetics. In ventricular myocytes, bPTH-(1-34) increased the amplitude, shifted the peak of the I-V relationship in the hyperpolarization direction and decreased the rate of L channel current activation. These effects of bPTH-(1-34) were hypothesized to be mediated by increased cAMP levels. When db-cAMP was applied to the bath solution, this non-hydrolysable but permeable cAMP analog mimicked every aspect of the effects of bPTH-(1-34) on L channel currents in both vascular and ventricular muscle cells. The present study demonstrated that cAMP at certain concentrations inhibited L channel currents when applied outside the plasma membrane of vascular smooth muscle cells but increased L channel currents in ventricular myocytes. However, at the present time, no conclusion can be drawn concerning the effect of various concentrations of intracellular cAMP on L channel currents.

2). No synergistic effect could be observed between db-cAMP and bPTH-(1-34). A non-synergistic effect, as seen here, is a common phenomenon when two agents use the same pathway. Submaximal responses are additive. As an analogy, the effects of isoproterenol and cAMP were also not synergistic (Kameyama *et al.*, 1985).

3). The antagonist of cAMP, Rp-cAMPs, completely abolished the effect of bPTH-(1-34) on L channels. Rp-cAMPs, a cAMP analogue with a sulphur atom substitution in the equatorial exocyclic oxygen position, binds to the cAMP-binding sites on cAMP-dependent protein kinase with a 90-fold increased binding affinity compared with cAMP (Rothermel and Bolelho, 1988). The binding of Rp-cAMPs to the regulatory subunits does not induce the dissociation of the holoenzyme and, hence, competitively inhibits the cAMP-induced activation of the cAMP-dependent protein kinase (protein kinase A). In my experiments, bPTH-(1-34) had no effect on L channel currents in ventricular myocytes when Rp-cAMPs was included in the pipette. This result indicates that the activation of cAMP-dependent protein kinase is necessary for the PTH-induced increase in L channel currents in ventricular myocytes. A similar situation was reported for the histamine-elicited increase in calcium channel currents in cardiac myocytes (Hescheler *et al.*, 1987a). Rp-cAMPs antagonised the effect of bPTH-(1-34) on L channel currents in vascular smooth muscle cells. If the same logic used for ventricular myocytes were used here, bPTH-(1-34) should also exert its effect via the activation of cAMP-dependent protein kinase, which, in turn, should result in an increase in the L channel current in vascular smooth muscle cells. However, bPTH-(1-34) inhibits, instead of activates, L channel currents in vascular smooth muscle cells. Does bPTH-(1-34) inhibit L channel currents in vascular smooth muscle cells by increasing the cAMP level and then activating cAMP-dependent protein kinase? How can the antagonistic effect of Rp-cAMPs on bPTH-(1-34)-induced inhibition of L channel currents in vascular smooth muscle cells be explained? An attempt is made in the next paragraph to answer these questions.

2. Two target protein kinases of cAMP.

It has been reported that an increase in cAMP content in vascular smooth muscle cells may have either of two effects, i.e. an increase or decrease in intracellular calcium concentration (Lincoln *et al.*, 1990). These contradictory results were ascribed to the activation of different protein kinases by cAMP. A decrease in intracellular Ca^{++} can be seen if cGMP-dependent protein kinase is activated by cAMP. On the other hand, activation of cAMP-dependent protein kinase by cAMP would result in increased entry of extracellular Ca^{++}. The presence of both cAMP- and cGMP-dependent protein kinases in primary cultured vascular smooth muscle cells has been demonstrated (Lincoln *et al.*, 1990). Hence, the effect of bPTH-(1-34) on L channel currents in this cell preparation could be the result of the predominant activation of cGMP-dependent protein kinase, which was secondary to the increase in the cAMP content stimulated by bPTH-(1-34). If Rp-cAMPs could bind competitively to cGMP-dependent protein kinase the inhibition of the effect of bPTH-(1-34) on L channel currents by Rp-cAMPs would be explained. Such binding has not, however, been reported. The application of specific inhibitors of cGMP-dependent protein kinase, such as Rp-cGMPs, will provide more information about the mechanism of cAMP-mediated PTH effects on L channel currents in vascular smooth muscle cells.

On the other hand, cGMP-dependent protein kinase was absent or the concentration was too low to be routinely detectable in cardiac and many other tissues (Lincoln *et al.*, 1976, 1981; Walter, 1981; Reuter, 1983). Furthermore, cGMP had no effect on calcium channel currents in single ventricular cells (Hartzell and Fischmeister, 1986). Although a more potent analogue of cGMP, 8-bromo-cGMP, has been reported to inhibit the calcium channel current in embryonic chick ventricular myocytes (Wahler *et al.*, 1990), this inhibitory effect could be interpreted as an increased cAMP hydrolysis via a cGMP-stimulated cyclic nucleotide phosphodiesterase (Flitney and Singh, 1981; Martins *et al.*, 1982; Lincoln and Johnson, 1984; Weishaar *et al.*, 1985). In this regard, the influence of cAMP on cGMP-dependent protein kinase in cardiac tissue would be negligible. In the present study, when cAMP concentration was increased by bPTH-(1-34), it probably only activated cAMP-dependent protein kinase in cardiac tissues, and, hence, an increase in L channel currents in ventricular myocytes occurred. This hypothesis needs more experimental evidence and at this point is only speculation.

In the presence of Rp-cAMPs in the pipette solution, bPTH-(1-34) failed to inhibit L channel currents in vascular smooth muscle cells, and failed to increase L channel currents in ventricular myocytes. In fact, small increases or decreases in L channel currents in vascular smooth muscle cells and ventricular myocytes, respectively, by bPTH-(1-34) were observed with Rp-cAMPs dialysis. Rp-cAMPs appears to reverse the PTH effect in both cells type. These changes in L channel currents, however, were not statistically significant. This result at least suggests that another signal transduction pathway in addition to the cAMP pathway may be involved in the PTH effect on calcium channels. For instance, the calcium and phospholipid-dependent protein kinase C (PKC) system appears to play an important role in mediating hormonal effects in different cell preparations. Activation of PKC by phorbol esters or diacylglycerols inhibited the peak calcium channel currents in N1E-115 cells (Linden and Pouttenberg, 1989), but increased intracellular calcium in osteosarcoma cells (Yamaguchi *et al.*, 1987) and L channel currents in the $A7_r5$ cultured vascular smooth muscle cell line (Fish *et al.*, 1988). A decrease in the contraction amplitude has been observed in phorbol ester-treated neonatal rat ventricular myocytes (Dösemeci *et al.*, 1988). Further studies by Lacerda *et al.* (1988) demonstrated that exposure to phorbol ester for a relatively long time period (20 min) significantly inhibited L channel currents. On the other hand, it has also been demonstrated that PTH translocated PKC from the cytosol to the membrane and increased the membrane-bound to cytosolic activity ratio of PKC by a factor of 2.2 in osteosarcoma cells (Abou-Samara *et al.*, 1989; Iida-Klein *et al.*, 1989). In cultured kidney tubule cells, PTH increased intracellular thiol proteinase activity, which was mimicked by phorbol esters but antagonized by the calcium ionophore A23187. When the PKC inhibitor, staurosporine, was applied, the PTH effect was abolished in this cell preparation (Denek *et al.*, 1990). Pretreatment of osteosarcoma cells with PTH abolished the phorbol ester-induced increase in free intracellular calcium (Yamaguchi *et al.*, 1987). Taken together, these observations suggest that PTH may activate PKC which in turn modulates calcium channels in different cell preparations. If this is the case, the Rp-cAMPs-induced reversal of the PTH effect on L channel currents could be explained as follows. PTH may activate PKC and increase intracellular cAMP levels. The latter has a predominant effect on the modulation of L channel currents. After the cAMP pathway is blocked by Rp-cAMPs, the PKC effect on L channel currents in different cell preparations will be unmasked. However, no information regarding the activation of PKC by PTH in vascular smooth muscle cells or neonatal rat ventricular myocytes is available.

In conclusion, cAMP decreases L channel currents in vascular smooth muscle and increases them in ventricular muscle cells, respectively, under the present experimental conditions. The effect of PTH on L channel currents in smooth muscle and ventricular muscle cells is mediated by cAMP although the target kinases of cAMP pathway appear to be different. The mechanisms by which increased cAMP content in smooth muscle and ventricular muscle cells modulated L channel currents differentially deserves further exploration.

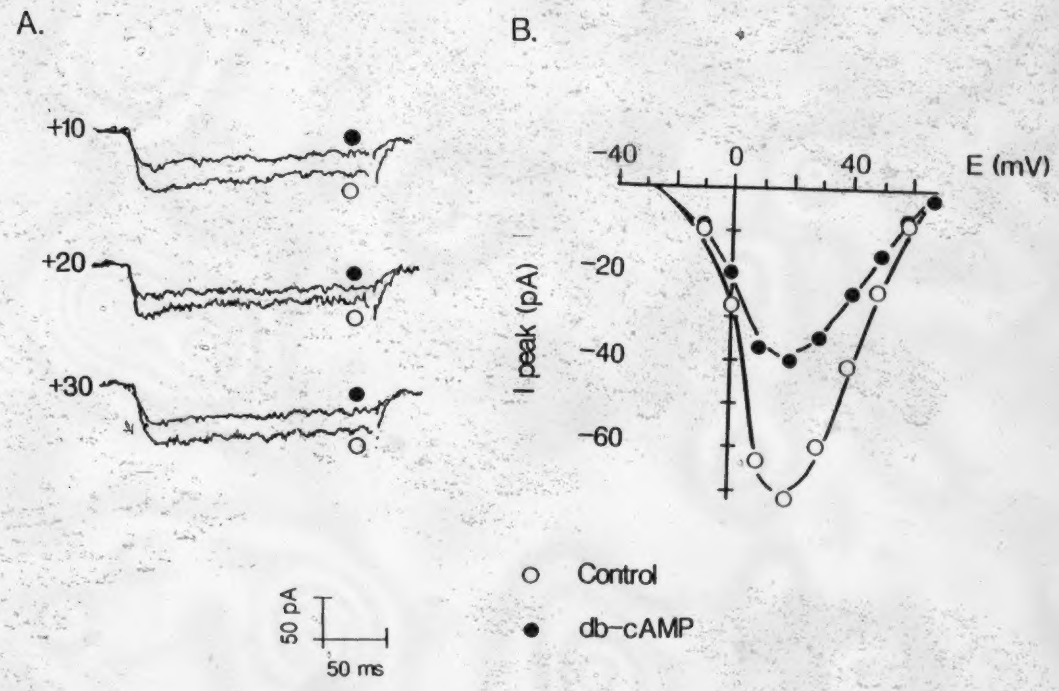

A.
+10
+20
+30
●
○
50 pA
50 ms
B.
−40
0
40
E (mV)
−20
−40
−60
I peak (pA)
○ Control
● db-cAMP

Fig. X-1 The effect of db-cAMP on L channel currents in a vascular smooth muscle cell. L channel currents were elicited from a holding potential of -40 mV to various test pulses. Leakage and capacitive currents have been corrected. 1 mM db-cAMP decreased the amplitude of L channel currents. **A.** The original current traces in the absence and then presence of db-cAMP were obtained at the various test pulses indicated beside each record. **B.** The I-V relationships of L channels before and after the application of db-cAMP.

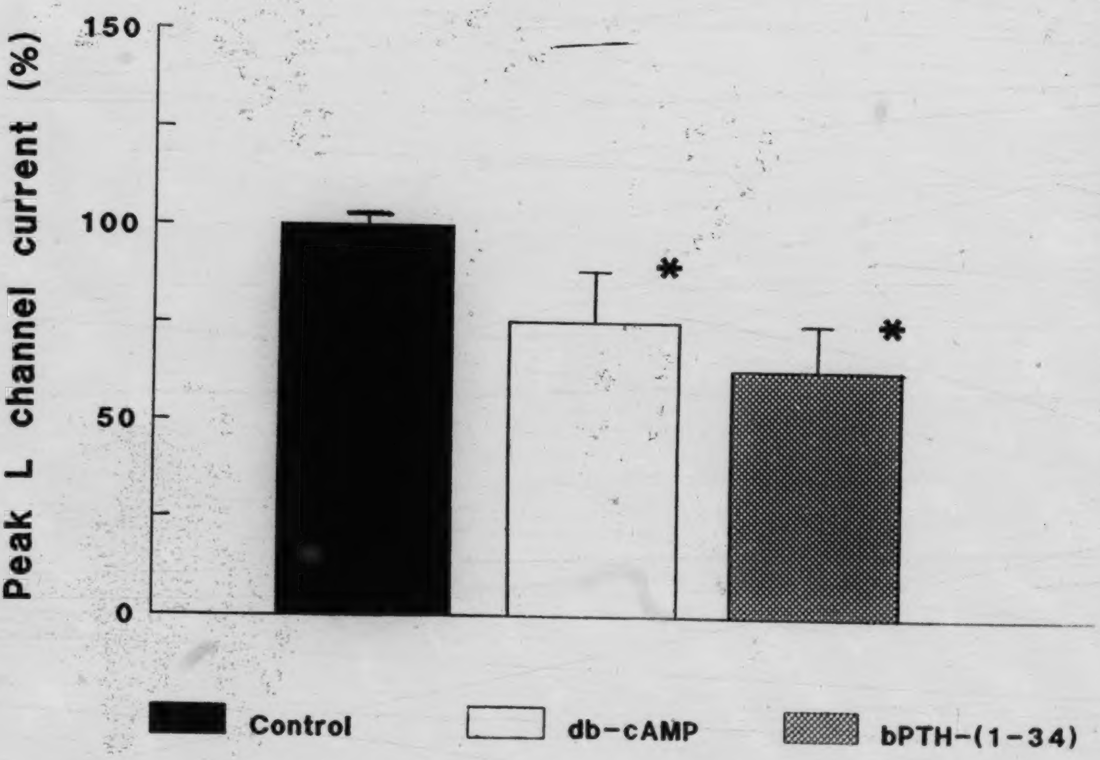

Peak L channel current (%)
150
100
50
0
*
*
Control
db-cAMP
bPTH-(1-34)

Fig. X-2 The effect of sequential application of db-cAMP and bPTH-(1-34) on L channel currents in vascular smooth muscle cells. db-cAMP at 1 mM decreased the amplitude of L channel currents by 25%. After the effect of db-cAMP on L channel currents was established, 1 μM bPTH-(1-34) was added to the bath. The amplitude of L channel currents was decreased by an additional 24% by bPTH-(1-34) ($n=5$, $p<0.05$).

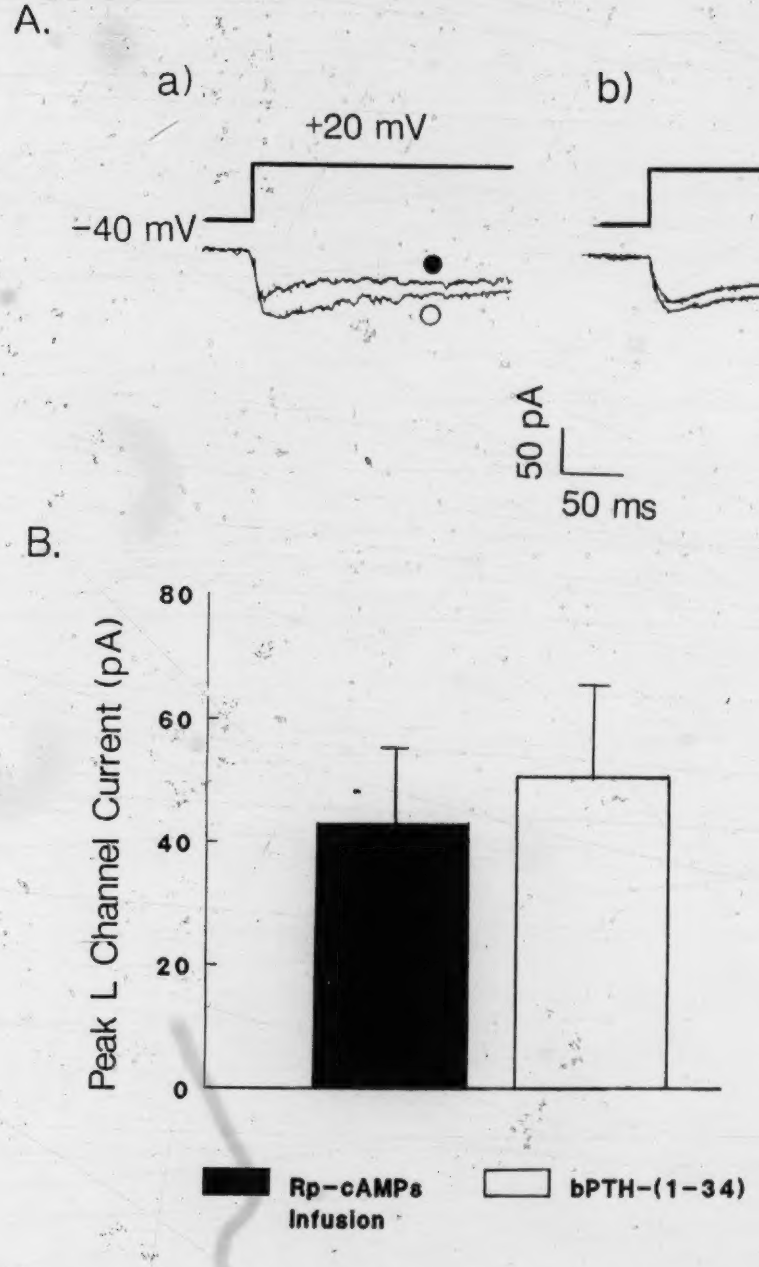

A.
a)
b)
+20 mV
−40 mV
●
○
50 pA
50 ms
B.
Peak L Channel Current (pA)
80
60
40
20
0
Rp-cAMPs Infusion
bPTH-(1-34)

Fig. X-3 Modification of bPTH-(1-34) effects by the intracellular infusion of Rp-cAMPs in vascular smooth muscle cells. **A.** The original current traces in the absence (open circles) and then presence (filled circles) of 1 μM bPTH-(1-34). The records in **A-a)** were taken from a cell (RJY1502) without Rp-cAMPs in the pipette solution. The records in **A-b)** were taken from another cell (M0608) with Rp-cAMPs in the pipette solution. **B.** With 100 μM Rp-cAMPs in the pipette, the effect of bPTH-(1-34) on L channel currents was abolished ($n=5$, $p>0.05$).

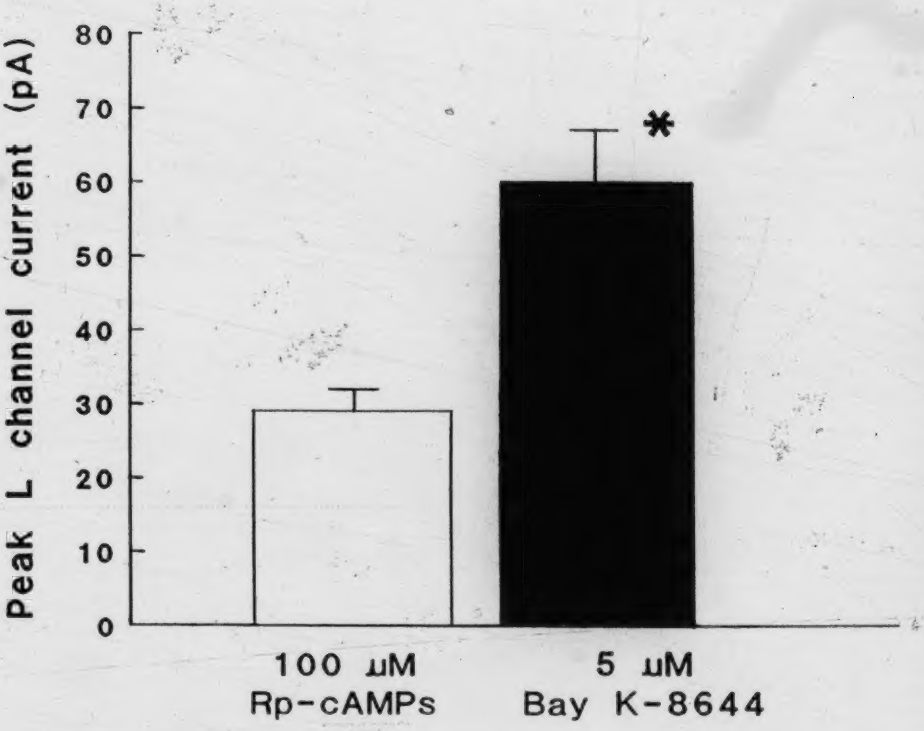

Peak L channel current (pA)
80
70
60
50
40
30
20
10
0
*
100 µM
Rp-cAMPs
5 µM
Bay K-8644

Fig. X-4 The effect of Bay K-8644 on L channel currents in the presence of Rp-cAMPs in vascular smooth muscle cells. When Rp-cAMPs was present in the pipette solution, Bay K-8644 still increased the amplitude of L channel currents in vascular smooth muscle cells ($n=4$, $p<0.05$).

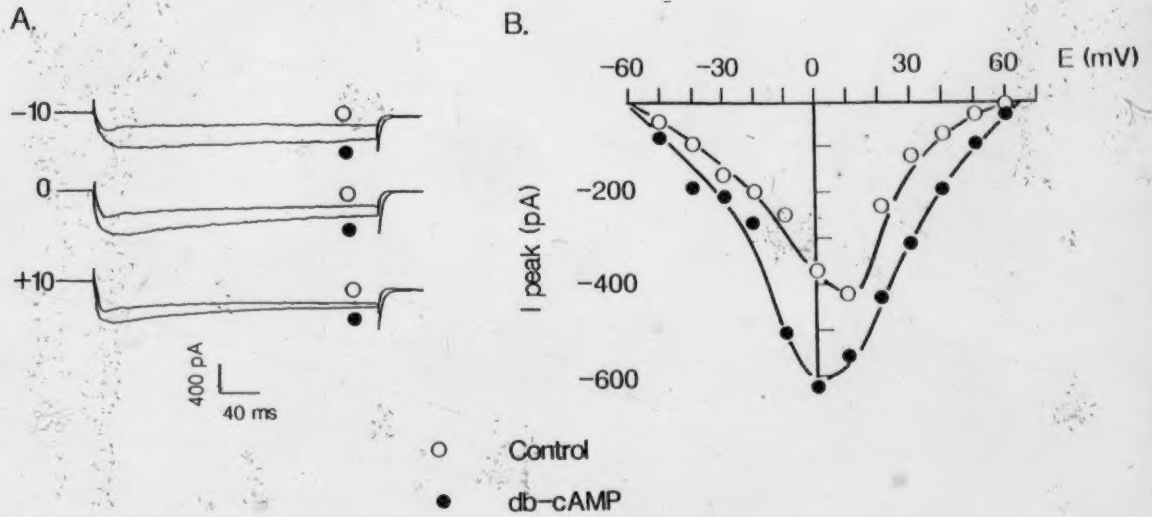

A.
−10
0
+10
400 pA
40 ms
B.
−60
−30
0
30
60
E (mV)
−200
−400
−600
I peak (pA)
○ Control
● db−cAMP

Fig. X-5 The effect of db-cAMP on L channel currents in one ventricular myocyte. L channel currents were elicited from a holding potential of -40 mV to various test pulses. Leakage and capacitive currents have been corrected. 1 mM db-cAMP increased the amplitude of L channel currents. A. The original current traces in the absence and then presence of db-cAMP were obtained at various test pulses indicated beside each record. **B.** The I-V relationships of L channel currents before and after the application of db-cAMP.

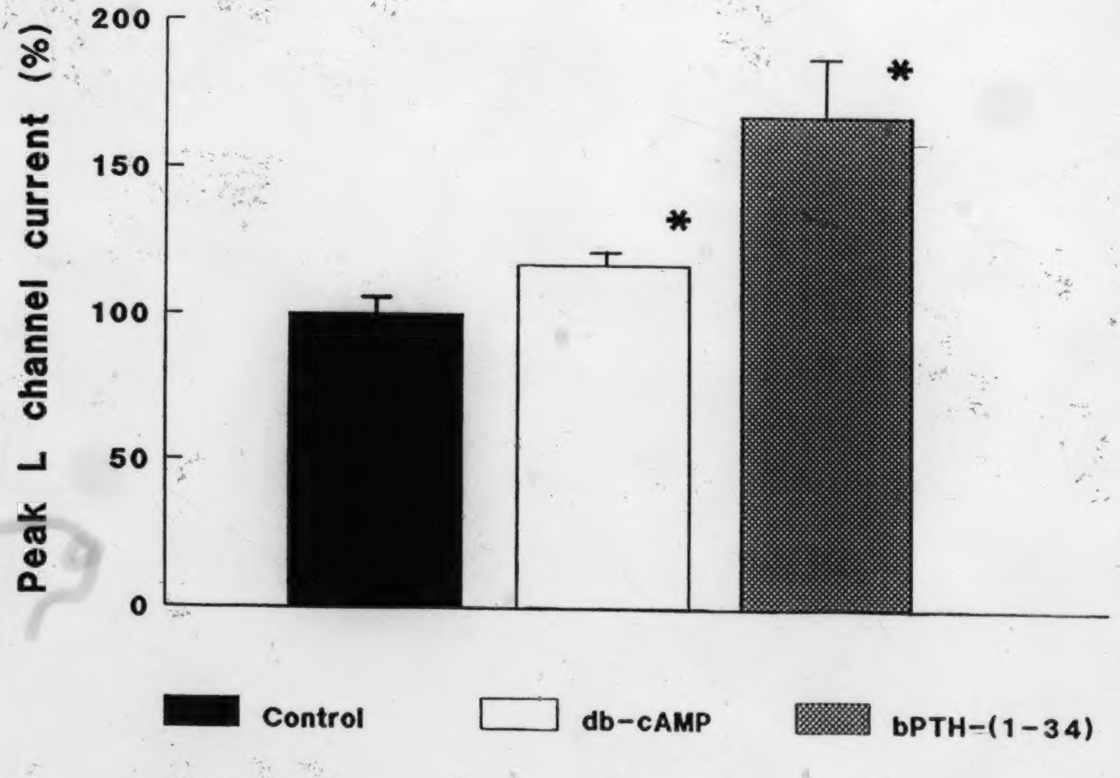

Peak L channel current (%)
200
150
100
50
0
*
*
Control
db-cAMP
bPTH-(1-34)

Fig. X-6 The effect of sequential application of db-cAMP and bPTH-(1-34) on L channel currents in ventricular myocytes. db-cAMP at a concentration of 1 mM increased the amplitude of L channel currents by 17%. After the effect of db-cAMP on L channel currents was established, 1 μM bPTH-(1-34) was added to the bath. bPTH-(1-34) increased the amplitude of L channel currents by an additional 51% ($n=5$, $p<0.05$).

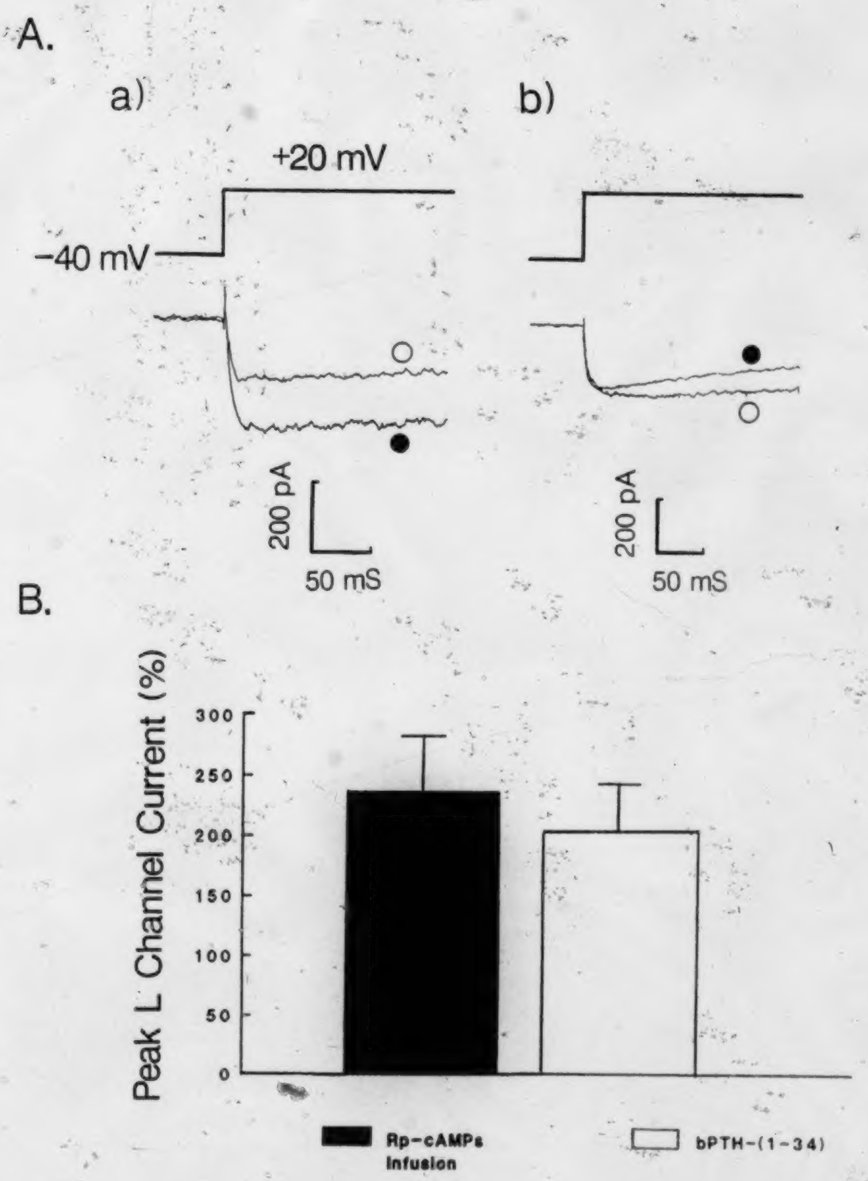

A.
a)
b)
+20 mV
−40 mV
○
●
200 pA
50 mS
B.
Peak L Channel Current (%)
300
250
200
150
100
50
0
Rp-cAMPs Infusion
bPTH-(1-34)

Fig. X-7 Modification of bPTH-(1-34) effects by the intracellular infusion of Rp-cAMPs in ventricular myocytes. **A.** The original current traces in the absence (open circles) and then presence (filled circles) of 1 μM bPTH-(1-34). The records in **A-a)** were taken from a cell (RWD30J2) without Rp-cAMPs in the pipette solution. The records in **A-b)** were taken from another cell (A0510) with Rp-cAMPs in the pipette solution. **B.** With 100 μM Rp-cAMPs in the pipette solution, the effect of bPTH-(1-34) on L channel currents was abolished ($n=7$, $p>0.05$).

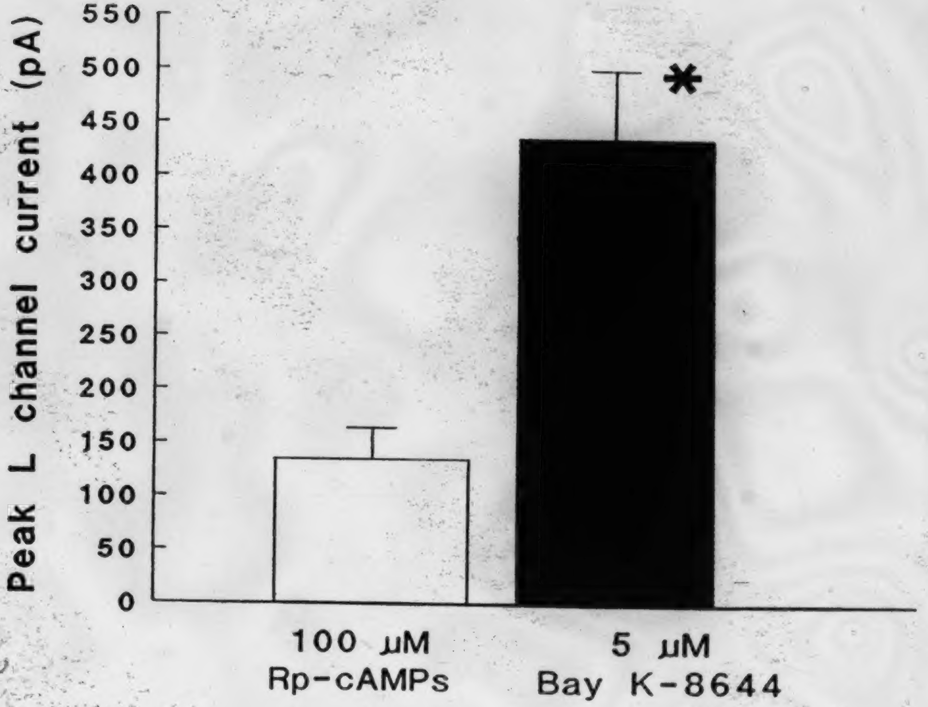

Peak L channel current (pA)
550
500
450
400
350
300
250
200
150
100
50
0
*
100 µM
Rp-cAMPs
5 µM
Bay K-8644

Fig. X-8 The effect of Bay K-8644 on L channel currents in ventricular myocytes in the presence of Rp-cAMPs. When Rp-cAMPs was present in the pipette solution, Bay K-8644 still increased the amplitude of L channel currents in ventricular myocytes ($n=5$, $p<0.05$).

CHAPTER 11

Summary and General Discussion

[SUMMARY]

1. The similarities and differences between two types of voltage- and time-dependent calcium channel currents in neuroblastoma, vascular smooth muscle and ventricular muscle cells:

Two types of voltage- and time-dependent calcium channel currents have been identified in neuroblastoma, vascular smooth muscle and ventricular muscle cells. These currents have many similar characteristics in the three cell preparations in terms of electrophysiological behaviour and pharmacological sensitivity. There are also differences in these channel currents in different tissues.

T channel currents are, basically, the same in all three cell preparations. In neuroblastoma cells, T channel currents are mainly expressed in the early stage of differentiation (Yoshii *et al.*, 1988). The ventricular myocytes used in the present study are from neonatal rats, in which T channel currents are readily measurable. T channel currents in adult ventricular myocytes have a lower density in single cells and a smaller population than do L channel currents (Bean, 1989a). In contrast, T channel currents are approximately twice as large as L channel currents in embryonic chick ventricular cells (Kawano and DeHaan, 1989a,b). These developmental changes in the expression of T channel currents in neuroblastoma and ventricular cells may be related to the function of T channel currents. The electrical excitation function of sodium channels in neuronal and cardiac tissues is well documented. Since the potential range of activation of sodium and T-type calcium channels overlap, the electrical function of T channels, i.e. depolarization, in these tissues may not be necessary at later stages of development. However, in addition to depolarization, the other functions of T channels and their correlation with sodium channels in the early stages of development are unknown.

The situation is different in vascular smooth muscle cells. T channel currents are expressed in vascular smooth muscle cells, including cells from rat tail artery. With the exception of one report (Sturek and Hermsmeyer, 1986), sodium channels have not been demonstrated in vascular smooth muscle cells. Therefore, the existence of T channel currents may provide the mechanism for the slow spontaneous action potentials in this cell preparation. In this regard, it can be postulated that the population and density of T channel currents are related to the development and function of different types of cells. This hypothesis is also in line with the statement in Bean's review (1989a) that the "T current is especially prominent in embryonic and immature cells, raising the possibility..." that it is important at an early stage of development.

When compared to T channel currents, L channel currents in different tissues show more variation. In N1E-115 cells, L channel currents with 20 mM Ba^{++} as the charge carrier did not inactivate during a time period of 200 ms from 0 to +30 mV. However, this current under the same experimental conditions inactivated slowly in both vascular smooth muscle and ventricular muscle cells. Although L channel currents were sensitive to dihydropyridines in all three cell preparations, Bay K-8644 shifted the peak of the I-V relationships towards more negative membrane potentials and increased the kinetics of inactivation in vascular smooth muscle cells and ventricular myocytes, but not in neuroblastoma cells. These results suggest that the gating mechanisms by which Bay K-8644 modulates L channel currents are different. The similarities and differences of T and L channel currents in different cell preparations are further summarized in Tables XI-1 and XI-2.

T and L channels characterized in this thesis are based on the electrophysiological and pharmacological characteristics of each channels. This classification of calcium channels will facilitate further studies and is necessary. However, it should be pointed out that this classification is over-simplified under some circumstances. Data from single channel recording is needed to determine the conductance of each type of calcium channels. More specific pharmacological tools are needed to separate L, T, N and other unidentified calcium channels. Even within the scope of this thesis, some parameters used to characterize different types of calcium channels are not very satisfactory, and need to be carefully considered.

For example, the normalized conductance curve, or activation curve, for calcium channels may not accurately reflect the conductance of a specific calcium channel. Among many reasons for this are the nonlinear instantaneous current-voltage relationship of calcium channels, the difficulty in determining the reversal potential for calcium channel currents due to the huge concentration difference of extracellular and intracellular free calcium and the efflux of Cs^+ through calcium channels, and the contribution by the opening of other types of ionic channels to the membrane conductance which was assumed to represent only calcium conductance. Therefore, the normalized conductance curve only provides a quantitative indication of the relative conductance of calcium channels.

Table XI-1:

Characteristics of T channel currents in three cell preparations

	Neuroblastoma Cells (N1E-115)	Vascular Smooth Muscle Cells	Ventricular Myocytes
Activation Threshold (mV)	-50	-50	-50
Maximum Activation Potential (mV)	-20	-10	-10
Reversal Potential (mV) (apparent)	+50	+50	+50
Peak Amplitude of Currents (pA)	100 - 400	20 - 80	50 - 200
Half steady-state Inactivation Potential (mV)	-60	-50	-75 (1)
Inactivation Kinetics	Fast Inactivation	Fast Inactivation	Fast Inactivation
Bay K-8644	No effect	No effect	No effect
Nifedipine	No effect	No effect	No effect
La^{+++}	Block	Block	Block

Note: (1): Data was adopted from Hagiwara *et al.*, 1988.

Table XI-2:

Characteristics of L channel currents in three cell preparations

	Neuroblastoma Cells (N1E-115)	Vascular Smooth Muscle Cells	Ventricular Myocytes
Activation Threshold (mV)	-20	-20	-20
Maximum Activation Potential (mV)	+20	+20	+20
Reversal Potential (mV) (apparent)	+50	+50	+50
Peak Amplitude of Currents (pA)	100 - 300	20 - 100	100 - 1000
Half steady-state Inactivation Potential (mV)	-13 (1)	-30	-5
Half Maximum Conductance Potential (mV)	(-)	0	+5
Inactivation Kinetics	Minimum inactivation	Moderate inactivation	Slow inactivation
Bay K-8644	Increase	Increase	Increase
Nifedipine	Decrease	Decrease	Decrease
La^{+++}	Block	Block	Block

Note: (1): Data was adopted from Ogata and Narahashi, 1990.

(-) indicates that no experimental data are available.

2. PTH effects on L channel currents are different in different cell preparations:

T channel currents in different types of cells were insensitive to PTH peptides. L channel currents were modulated differentially by bPTH-(1-34) in various cell preparations. The modulation of L channel currents by bPTH-(1-34) included changes in the steady-state activation and inactivation, potential dependency of the PTH effect, kinetic changes, etc (Table XI-3). The enhancement and inhibition of L channel currents in vascular smooth muscle cells and ventricular myocytes, respectively, in the presence of bPTH-(1-34) can be functionally related to the morphological changes of corresponding cell preparations (Table XI-4). The close relationship of the PTH effect on L channel currents and the morphological changes suggests that the L channel current plays an important role in the contraction of both vascular smooth muscle cells and ventricular myocytes. Furthermore, the morphological studies presented in this thesis indicate that the results of the PTH effect on calcium channels under the present experimental conditions have functional relevance.

Table XI-3:

The modulation of L channel currents in three cell preparations by bPTH-(1-34)

	Neuroblastoma Cells (N1E-115)	Vascular Smooth Muscle Cells	Ventricular Myocytes
Maximum Activation Potential	No shift	No shift	Negative shift
Peak Current Amplitude	Decrease	Decrease	Increase
Potential-dependent	(-)	Yes	No
Concentration-dependent	Yes	Yes	Yes
Normalized Conductance	(-)	No change	Negative shift
Activation Rate	No change	No change	Decrease
Inactivation Rate	No change	No change	No change
Antagonistic action of bPTH-(3-34)	(-)	Yes	Yes
H_2O_2-bPTH-(1-34)	No effect	(-)	(-)
Wash-out (within 5 min)	(-)	Reversible	Irreversible
Bay K-8644 reversal	Yes	Yes	(-)
Nifedipine reversal	(-)	(-)	Yes
La^{+++} block	Yes	Yes	Yes

Note: (-) indicates experimental data are unavailable.

Table XI-4:

The correlation of morphological changes with modulation of

L channel currents induced by bPTH-(1-34)

	Vascular Smooth Muscle Cells	Ventricular Myocytes
L channel currents	Decrease	Increase
Cell contraction	Relaxation	Contraction
Spontaneous beating	(-)	Increase

3. PTH effects on L channel currents in vascular smooth muscle cells and ventricular myocytes are mediated by cAMP.

The effects of bPTH-(1-34) on L channel currents in vascular smooth muscle cells and ventricular myocytes can be mimicked by db-cAMP (Table XI-5). Furthermore, the effects of bPTH-(1-34) and db-cAMP on L channel currents are additive but not synergistic. In addition, both the inhibition and enhancement of

L channel currents by bPTH-(1-34) in vascular smooth muscle cells and ventricular myocytes, respectively, can be eliminated by the intracellular perfusion of Rp-cAMPs, a cAMP antagonist. All these results point to the conclusion that cAMP is the main second messenger mediating PTH effects on L channel currents in vascular smooth muscle cells and ventricular myocytes.

Table XI-5:

The mediation of bPTH-(1-34) effects on L channel currents in vascular smooth muscle cells and ventricular myocytes

		Vascular smooth muscle cells	Ventricular myocytes
Peak amplitude	bPTH-(1-34)	Decrease	Increase
	db-cAMP	Decrease	Increase
I-V relationship	bPTH-(1-34)	No shift	Shift
	db-cAMP	No shift	Shift
Activation Rate	bPTH-(1-34)	No change	Decrease
	db-cAMP	No change	Decrease
Addition of the effects of cAMP and bPTH-(1-34)		Yes	Yes
Antagonization of bPTH-(1-34) effect by Rp-cAMPs		Yes	Yes

1. bPTH-(1-34) has different effects on L channel currents in different tissues:

In this thesis, bPTH-(1-34) has been shown to affect L, but not T, channel currents in three different cell preparations. The modulation of L channel currents by bPTH-(1-34) can be related to cell contraction (smooth muscle cells and ventricular myocytes) and spontaneous beating of ventricular myocytes in response to a PTH challenge. It is interesting that the effects of bPTH-(1-34) on L channel currents have tissue selective features. In neuroblastoma and vascular smooth muscle cells, bPTH-(1-34) inhibited L channel currents. However, bPTH-(1-34) increased L channel currents in ventricular myocytes. This selective action of PTH on L channel currents in different cell preparations provides the mechanism for PTH-induced vasorelaxation and the cardiotonic effect. The tissue-selective effect of PTH may not only be important in the physiological regulation of the cardiovascular system, but may also have pharmacological significance in that modified forms of PTH may be of use in cardiovascular diseases. For example, the clinical use of calcium agonists, such as Bay K-8644, have been suggested in the treatment of cardiac failure. However, in addition to the desired positive inotropic effect, vasoconstriction and, in turn, hypertension are likely (Porzig, 1989). Furthermore, serious central nervous system side effects are often seen after the application of calcium channel agonists. These side effects are related to the activation of L channels in the CNS associated with neurotransmitter release (Bechem *et al.*, 1988). In contrast, PTH produced a positive inotropic effect (excitation of L channels) and vasorelaxation (inhibition of L channels) as well as inhibition of neuronal activity (inhibition of L channels). Hence, the possibility of using modified forms of PTH, therapeutically, is attractive.

There are several factors which may explain the tissue specificity of the PTH effects on L channel currents.

If the PTH receptor is a part of the L channel protein complex, PTH might bind directly to L channel proteins, as is the case with TTX blockage of sodium channels (Narahashi *et al.*, 1964; Sigworth, 1980). This also occurs in the endplate channel gating in which two acetylcholine molecules binding on one endplate channel directly opened the channel (Karlin, 1980; Conti-Tronconi and Raftery, 1982). However, this analogy cannot be extended to include the PTH effect on L channel currents. The reasons are as follows. 1). PTH cannot completely inhibit L channel currents. 2). PTH action on L channel currents is slow in onset (5 min in vascular smooth muscle cells and more than 10 min in ventricular myocytes). A similar situation has been reported regarding the endothelin effect on calcium channel currents in vascular smooth muscle cells, in which a period of more than 5 min was required to establish a stable action (Inoue *et al.*, 1990) after the application of endothelin to the bath. 3). After the effect of some intracellular second messengers, such as cAMP, were completely inhibited, the PTH effect on

L channel currents was eliminated. To obtain a clear picture of the direct effect of PTH on L channels more evidence is needed. For example, it is important to know whether PTH can bind directly to L channels and whether the PTH effect on L channel currents can still be detected in cell-free patches.

The variability of the L channel proteins in different cell preparations is another possibility. As is summarized in this Chapter, L channel currents have not only similar, but also different, characteristics in different tissues. If the same PTH receptors are coupled to different L channel proteins or their closely related regulatory proteins, the binding of PTH may affect these different subtypes of L channels by inhibition or excitation in different tissues. Pharmacological studies have revealed differences between neuronal and non-neuronal L channels. For example, ω-conotoxin blocked L channels in neurons but not in cardiac, skeletal or smooth muscle cells (Hofmann *et al.*, 1987). Biochemical evidence also implies the existence of tissue specific isoforms of L channels (dihydropyridine receptors) (Hofmann *et al.*, 1987). Different α_1 subunits of dihydropyridine receptors have been isolated from skeletal muscle, cardiac muscle (Slish *et al.*, 1989), brain and aorta (Koch *et al.*, 1989). Although some differences in the electrophysiological characteristics of L channel currents in three different cell preparations have been reported in the present study, no definite conclusion regarding the tissue-selective variation of L channels can be drawn at this time.

On the assumption that L channel proteins have the same molecular structures, tissue-selective distribution of the two types of PTH receptors would be partly responsible for the tissue-selective effect of PTH on the same L channels. This is discussed in detail in Chapter 8.

If L channels are similar in molecular conformation and a single population of PTH receptors occurs in different cell preparations, the only possibility remaining for the tissue selective effects of PTH on L channel currents would be that the binding of PTH induces different intracellular events which modulate L channels in a tissue-specific way. Some of the evidence supporting this hypothesis includes: different onset rates of PTH effects, different effects of cAMP on L channel currents in different cell preparations, and the wash-out experiments in which the bPTH-(1-34) effect on L channel currents is readily reversible in vascular smooth muscle cells, but not in ventricular myocytes.

PTH binding might modulate L channel currents directly via different G proteins in different tissues. The PTH effect on G proteins has been reported in several cases (Teitelbaum *et al.*, 1982; Babich *et al.*, 1989). Since the PTH effect on G protein activities and the G protein effect on L channels in neuroblastoma, vascular smooth muscle and neonatal rat ventricular muscle cells are unknown, it is difficult to speculate regarding this possibility. In addition, since a large component of the PTH effect is slow in onset, direct modulation of the Ca^{++} channel by G proteins cannot be the complete answer.

Alternatively, PTH binding may, initially, activate second messengers, which in turn cause differential modulation of L channel currents in various tissues. cAMP was initially chosen as such a candidate. The data presented in this thesis demonstrate that cAMP is the mediator of the PTH effect on L channel currents in rat tail artery smooth muscle cells and neonatal rat ventricular myocytes. bPTH-(1-34) induced the increase in cAMP levels, in which a G protein (G_s) may be the coupling factor. Increased intracellular cAMP levels activate different protein kinases in different cell preparations. This hypothesis may explain the opposite effect of PTH on L channel currents in different cell preparations. In primary cultured vascular smooth muscle cells, the bPTH-(1-34) induced increase in cAMP content may activate both cAMP-dependent protein kinase (protein kinase A) and cGMP-dependent protein kinase (protein kinase G) (Lincoln *et al.*, 1984, 1990). The net result of the activation of two protein kinases is the inhibition of L channel currents in vascular smooth muscle cells. However, the bPTH-(1-34) induced increase in cAMP content in neonatal rat ventricular myocytes may only activate protein kinase A since protein kinase G is absent in this tissue (Lincoln *et al.*, 1976, 1981). Hence, the cardiac L channel would be phosphorylated and the channel current increased. A detailed discussion of the cAMP effect on cAMP-dependent and cGMP-dependent protein kinases may be found in Chapter 10.

2. The comparison between PTH binding studies and PTH effects on L channel currents:

PTH receptors have been demonstrated in neuronal (Pang *et al.*, 1990) and rabbit renal microvessel smooth muscle cell membranes (Nickols *et al.*, 1990). However, the K_d for bPTH-(1-34) binding in neuroblastoma cells (3.7 nM, Pang *et al.*, 1990) seems much lower than the half effective concentration (around 1 μM) required to affect Ca^{++} channels in this cell preparation. This thousand-fold concentration discrepancy between the patch clamp and binding studies could cast some doubt on whether the responses obtained are actually due to the binding of the agents used to the PTH receptors. The following points may provide clues for this discrepancy. 1). In the binding studies, the synthetic bPTH-(1-34) analog, [$Nle^{8,18}$, Tyr^{34}]bPTH-(1-34), was used. This PTH analog has been demonstrated to be more stable than the unsubstituted bPTH-(1-34) while retaining full biological activity (Horiuchi *et al.*, 1983; Rosenblatt, 1986). However, in patch clamp studies, the unsubstituted bPTH-(1-34) was used. 2). In the patch clamp study, the use of artificial intracellular and extracellular solutions would facilitate the recording of calcium channel currents but might decrease the PTH receptor density or the ligand affinity. Consequently, the occupation of PTH receptors under patch clamp conditions would require a higher concentration of PTH. 3). There is no evidence showing equal densities of PTH receptors and L channel proteins. Hence, there is no stoichiometric basis for comparing binding of PTH and changes in L channel currents. 4). The binding of PTH to its receptor is necessary, but not sufficient, for its effect on L channel currents. The modification of L channel currents is preceded by the binding of PTH to receptors, the activation of G proteins, the increase in cAMP concentration, the activation of target kinases of cAMP and the phosphorylation and dephosphorylation of L channel proteins. The same concentration of PTH cannot activate each of these steps in the signal transduction process to the same degree. On the whole, the concentration of a hormone or peptide required for the expression of an effector system cannot be extrapolated in a straightforward manner from its binding affinity.

3. Inconsistent cell response to PTH peptides:

In studies using voltage and patch clamp techniques, an inconsistent cell response, i.e. only a percentage of cells have a specific response, to either endogenous substances or synthetic organic compounds is often encountered (Droogmans *et al.*, 1987; Okabe *et al.*, 1987). It occurs in the present investigation of the effect of bPTH-(1-34) on L channel currents. There are many possible explanations for this: 1). The number of PTH receptors on vascular smooth muscle cells may be different (Huang and Rorstad, 1984). The smooth muscle cells used in the present study were isolated from along the whole length of rat tail artery. The density of PTH receptors may be different at different segments of the artery, as in the case of various densities of adrenergic nerve terminals along the length of the cerebral arterial system (Burnstock, 1977). Consequently, the L channel response to PTH elicited from various cells with high or low density of

PTH receptors may, quite reasonably, be different; 2). Minor differences in the isolation, either varied digestion time, agitation time, culture time etc., will damage the PTH binding sites on the cell membrane to different degrees; 3). Different source, lot and preparation of bPTH(1-34) may produce different activity; 4). Different proportions of L and T channels resulted in the varied responses to bPTH-(1-34), which can be judged by the subsequent application of Bay K-8644; 5). The responsiveness may be changed by the degree to which some second messengers, such as cyclic AMP, are washed out during whole-cell recording.

In neuroblastoma cells cultured in 2% DMSO, 3 of 13 cells were insensitive to bPTH-(1-34). A likely explanation is that the cells tested were in different stages of differentiation. In certain amphibian neurons, Ca^{++} inward currents occurred as a usual phenomenon at certain developmental stages, disappearing during the process of complete maturation (Baccaglini and Spitzer, 1977). The presence of PTH receptors and the functional status of L channel proteins might be different during the process of neuroblastoma cell differentiation. An alternative explanation would be that another sub-type of L channels, such as those in vascular smooth muscle cells (Worley *et al.*, 1986), exists in N1E-115 cells. One of these subtypes of L channels might be sensitive to dihydropyridines but not to PTH. No experimental evidence is available to support this hypothesis.

4. The significance of the present studies:

PTH induced many functional responses in either "classical" or "non-classical" target tissues. However, whether the change in L channel currents induced by PTH in "non-classical" target tissues are physiological is not clear. The physiological and pathophysiological actions of PTH on calcium channels are discussed separately in the following sections.

1). Physiological function of PTH:

i. PTH receptors have been demonstrated in neuroblastoma (Pang *et al.*, 1990) and vascular smooth muscle cell membranes (Nickols *et al.*, 1989c; Orloff *et al.*, 1989a,c).

ii. A non-parathyroid gland origin of PTH has been reported in the central nervous system. If PTH can also be localized in nerve terminals supplying blood vessel and cardiac muscle, a higher local concentration of PTH would be anticipated. However, this hypothesis lacks experimental evidence. A similar situation has been demonstrated for CGRP. The CGRP-immunoreactive nerve fibres have been observed in the smooth muscle layers of the intestine and some CGRP-immunoreactive fibres run along blood vessels in the mucosal layers (Ohtani *et al.*, 1989). CGRP-like immunoreactive fibres were also found in the second and third branches of the mesenteric artery (Kawasaki *et al.*, 1988). In cardiac muscle, CGRP-like immunoreactive nerves have been observed in atrial myocardium (Saito *et al.*, 1987; Ishikawa *et al.*, 1988).

iii. It has been estimated that the normal circulating concentration of PTH is approximately 10^{-10} M (Fischer *et al.*, 1974; Carnes *et al.*, 1980). In *in vitro* studies, the threshold concentration of bPTH-(1-34) for relaxing the rat tail artery strips constricted by KCl was approximately 10^{-8} M (Pang *et al.*, 1988). In the present study, the minimal concentration of bPTH-(1-34) required to inhibit L channel currents in a single smooth muscle cell was 10^{-7} M. In patch clamp studies, enzymatic treatment will damage the cell membrane to a different degree although this procedure is necessary to disperse single cells. It has been reported that in cell dialysis experiments the concentrations used to block or mimic the hormonal response were much higher than those used in biochemical studies (10^{-9} to 10^{-6} M) (Hescheler *et al.*, 1987). Furthermore, the artificial extracellular (bath) and intracellular (pipette) solutions used will facilitate the recording of calcium channel current, but may decrease the channel response to hormones or neurotransmitters. For example, the inhibitory effects of dopamine and noradrenaline on calcium channel currents were enhanced by a low extracellular calcium concentration (2 mM) but abolished by a high extracellular calcium concentration (20 mM) (Marchetti *et al.*, 1986). It is possible that a much lower concentration of bPTH-(1-34) will be sufficient to affect L channel currents in smooth muscle cells if the extracellular calcium concentration is lowered to 1 or 2 mM. However, at this concentration range it is very difficult to record the small size calcium channel currents. Bearing these considerations in mind, the concentrations of bPTH-(1-34) used in the present study may be related to physiological concentrations of PTH *in vivo*.

iv. Physiological effects of PTH on the homeostasis of plasma calcium levels are well established. PTH increases the plasma calcium on one hand. The contraction of smooth muscle cells will be enhanced by the increased concentration of extracellular calcium on the other hand. The inhibition of voltage-dependent calcium channels in the plasma membrane of vascular smooth muscle cells and, in turn, the relaxation of vascular smooth muscles induced by PTH could counteract the increased muscle contractility in the presence of higher plasma calcium level. The physiological role of PTH in regulating muscle sensitivity to plasma calcium is believed to occur in this way. Furthermore, PTH may also regulate neuronal and cardiac activities via modulation of voltage-dependent calcium channels under physiological conditions.

2). *Pathophysiological function of PTH:*

PTH-like proteins (PTHLPs), a family of tumor-derived hypercalcemic factors, have been found in recent years (Orloff *et al.*, 1989a). This group of proteins shares amino acid sequence homology with PTH (Broadus *et al.*, 1988), and binds to the same receptors as PTH does in bone and kidney (Nissenson *et al.*, 1988; Orloff *et al.*, 1989b). Mok *et al.* (1989b) reported that pretreatment of rat gastric smooth muscle with a high concentration of either PTHLP-(1-34) or PTH-(1-34) desensitized the tissue to a subsequent exposure to either peptide. Furthermore, human (h) PTH-(1-34) and PTHLP-(1-34) are equipotent in stimulating adenylate cyclase (Yates *et al.*, 1988). Taken together, these results indicated that both PTH and PTHLPs act on the same receptors and, possibly, elicit the same functional responses. There is no evidence that PTHLPs modulate calcium channels in neuronal, vascular smooth muscle and cardiac muscle cells. Research on the PTHLPs effect on calcium channels in these cell preparations will help to prove the relevance of the present study and reveal the endogenous modulation of calcium channels under some specific pathophysiological conditions.

A correlation between hypertension and hyperparathyroidism has been demonstrated for many years (Rosenthal and Roy, 1972; Scholz and Minn, 1977). However, PTH/PTHLPs are not the causative factor of hypertension. First, PTH dilates peripheral resistance vessels and lowers blood pressure. One mechanism in this process is the inhibition of calcium channel currents demonstrated in the present study. Second, after surgical correction of primary hyperparathyroidism, some patients have become hypertensive (Cirillo *et al.*, 1989). Third, patients with severe hypertension displayed excess production of PTHLPs (Mundy, 1987) as a compensation, possibly, for the increase in blood pressure (Nickols *et al.*, 1989a). Fourth, in stroke prone spontaneous hypertensive rats, acute i.v. administration of PTH-(1-34) decreased blood pressure in a concentration-dependent manner. Furthermore, in the same set of experiments, chronic administration of PTH-(1-34) s.c. for 4 weeks attenuated the increase in blood pressure (Neuser *et al.*, 1990).

The low serum ionized calcium concentration often detected in hypertension (Young *et al.*, 1988) stimulates the secretion of PTH. PTH, then, exerts its effects on the cardiovascular system. The inhibition of L channels in vascular smooth muscle cells is one of those PTH-induced beneficial compensatory effects. As a result, the elevated arterial blood pressure in hypertension is partially counteracted. In fact, PTH has been demonstrated to be more potent in eliciting vasorelaxation in spontaneously hypertensive rats than in normotensive animals (McCarron *et al.*, 1984).

In cardiovascular pathology, PTH/PTHLPs are suggested to play a modulatory role in controlling blood pressure and cardiac function (Mok *et al.*, 1989a). This modulatory function may be, in part, the modulation of calcium channels in cardiovascular tissues.

[CONCLUDING REMARKS]

T and L types of calcium channel currents have been identified in neuroblastoma cells, vascular smooth muscle cells and ventricular myocytes. These two channel currents have both homologous and heterologous characteristics. bPTH-(1-34) did not affect T channel currents in any of these three cell preparations. bPTH-(1-34) inhibited L channel currents in neuroblastoma and smooth muscle cells, but increased these currents in ventricular myocytes. The modulatory effect of bPTH-(1-34) on L channel currents could be antagonised by bPTH-(3-34) in vascular smooth muscle and ventricular muscle cells. Furthermore, cAMP is the mediator of the modulatory effect of bPTH-(1-34) on L channel currents in both vascular smooth muscle and ventricular muscle cells.

Voltage-dependent calcium channels provide an important pathway for Ca^{++} entry into cells. This entry triggers a series of biological activities. The present study increased our knowledge of the distribution and characterization of voltage-dependent calcium channels in N1E-115 cells, vascular smooth muscle cells and neonatal rat ventricular cells.

Although many synthetic compounds are currently available to open or close calcium channels for the purposes of experimental or clinical applications, it is still not quite clear how living organisms control their calcium channel activity under physiological or pathophysiological conditions. The present study provides evidence that PTH may be an endogenous modulator of voltage-dependent calcium channel currents in various tissues. Results derived from this thesis study concerning the different effects of PTH on calcium channel currents in various tissues and the selective actions of PTH on two types of calcium channel currents demonstrate the tissue specificity of PTH effects and have led to a more critical evaluation of the mechanism of PTH action. This study will also stimulate interest in the identification of other endogenous substances responsible for the modulation of calcium channels.

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APPENDIX

At the time of printing of this thesis some of its contents have been submitted for publication or published.

Some results from Chapters 3, 6 and 7 have been published in three journals.

Wang R, Karpinski E, Pang PKT 1990. Journal of Pharmacology and Experimental Therapeutics. 254(3):1006-1011.

Pang PKT, Wang R, Shan J, Karpinski E, Benishin CG 1990. Proceedings of the National Academy of Science. 87:623-627.

Pang PKT, Wang R, Wu LY, Karpinski E, Shan J, Benishin CG 1990. Experimental Gerontology. 25:247-253.

Some results from Chapter 4 have been published or accepted in two journals for publication.

Wang R, Karpinski E, Pang PKT 1989. American Journal of Physiology. 256(25):H1361-H1368.

Wang R, Karpinski E, Pang PKT 1990. Journal of Thermal Biology. In press.

Some results from Chapters 5 and 8 have been submitted for publication.

Wang R, Karpinski E, Pang PKT 1990. Journal of Cardiovascular Pharmacology.

Significant parts of the methods and discussions in above submitted, accepted and published papers have been incorporated in Chapters 2 and 11.